Alfredo G. Torres *Editor*

Escherichia coli in the Americas

Springer

Escherichia coli in the Americas

Alfredo G. Torres
Editor

Escherichia coli in the Americas

Springer

Editor
Alfredo G. Torres
Department of Microbiology and Immunology
University of Texas Medical Branch
Galveston, TX, USA

ISBN 978-3-319-45091-9 ISBN 978-3-319-45092-6 (eBook)
DOI 10.1007/978-3-319-45092-6

Library of Congress Control Number: 2016952245

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Foreword

In 1885, a German pediatrician, Theodor Escherich, first described the bacterium *Bacterium coli commune* as a normal intestinal inhabitant of healthy children. Although his research on this organism, subsequently named *Escherichia coli* in his honor, is largely remembered for the description of this species as a nonpathogenic, commensal intestinal inhabitant, he also reported in 1894 that *B. coli* was present in the urine of young girls suffering from urinary tract infections and suggested that it reached the bladder by the ascending route. This was the first description of *B.* (*E.*) *coli* as a potential cause of disease and was followed by Escherich's 1899 report that *B. coli* was the cause of dysentery. The latter report was preceded by Kiyoshi Shiga's 1898 report that the cause of dysentery was a bacterium that he called *Bacillus dysenteriae*, which was subsequently named *Shigella dysenteriae* in his honor. Conradi described a neurotoxin from lysates of this organism in 1903 which was later called Shiga toxin.

For several decades thereafter, no major advances were made in the study of pathogenic *E. coli* until the 1944 proposal by Kauffman of a scheme for serological classification of *E. coli* based on the O (somatic) antigen (a component of the lipopolysaccharide), the H (flagellar protein) antigen, and the K (acid polysaccharide capsular) antigen. The importance of this typing scheme cannot be exaggerated because it allowed the various strains of *E. coli* to be differentiated from one another. The development of this scheme allowed a British pediatrician, John Bray, to report in 1945 that antigenically homogeneous strains of *Bacterium coli neopolitanum* were associated with summer diarrhea in infants.

Although the serotyping scheme of Kauffman facilitated the differentiation of *E. coli* strains associated with disease from those strains not associated with disease, the virulence mechanisms remained unknown for many years. In 1956, De and colleagues in India demonstrated that *E. coli* isolated from adults and children with cholera-like illness caused fluid accumulation in ligated rabbit ileal loops. In England, Smith and Hall showed in 1967 that *E. coli* strains isolated from the stool of young animals with severe diarrhea could produce a heat labile (LT) and a heat stable (ST) enterotoxin and demonstrated that these traits were genetically controlled by plasmids. The development of tissue culture assays for LT and suckling mouse assays for ST eventually allowed the identification of these strains, which

were ultimately called enterotoxigenic *E. coli* (ETEC). Epidemiological studies in the 1960s and early 1970s in Brazil by Trabulsi and colleagues and in Bengal by Gorbach and Sack solidified the link between such strains and human diarrhea. Definitive proof that such strains were human pathogens was derived from volunteer challenge studies in the 1970s. ETEC is now known to be among the most common causes of infant diarrhea in developing countries and is the dominant cause of traveler's diarrhea. In the late 1970s, the Falkow laboratory ushered in the era of molecular pathogenesis by cloning the genes encoding LT and ST and developing DNA probes to diagnose strains possessing these genes. These studies reported the first virulence factor genes and diagnostic DNA probes for any microbial pathogen. Factors responsible for adherence to the small intestinal mucosa were discovered and characterized as fimbrial or fibrillar colonization factors (CFs or CFAs). More recent studies have identified additional accessory virulence factors for ETEC, and this continues to be an area of active research.

The serotypes recognized as ETEC differed from the first diarrheagenic *E. coli* serotypes recognized by Bray. Because the LT and ST toxins were plasmid-mediated, controversy arose in the field when some investigators claimed that strains belonging to the "classic" serotypes but lacking LT and ST had simply lost the plasmid-encoded virulence factors. This controversy was definitely resolved by challenge studies conducted by Levine and colleagues in which strains belonging to the classic diarrheagenic *E. coli* strains lacking LT and ST caused diarrhea in adult volunteers. The mechanisms by which these classic diarrheagenic *E. coli* strains, termed enteropathogenic *E. coli* (EPEC), caused diarrhea began to be elucidated by Kaper and colleagues who described a package of plasmid- and chromosomally encoded virulence factors that conspired to induce a so-called attaching-and-effacing lesion of the small intestine. A key set of virulence factors, including a type III secretion system, were shown to be encoded on a pathogenicity island called locus of enterocyte effacement (LEE). The aggressive outbreaks among infants in industrialized countries caused by EPEC have disappeared, but the pathogen continues to be an important cause of infant diarrhea in developing countries, particularly in sub-Saharan Africa. Questions that continue to confront EPEC research include the mechanism of its striking age-related pathogenicity, the contributions of its multiple virulence factors toward secretory diarrhea, and which of its factors may contribute to effective vaccine development.

In 1983, a new class of pathogenic *E. coli* was recognized from two landmarks but at first seemingly unrelated, epidemiological reports. Karmali and colleagues investigated an outbreak of hemolytic uremic syndrome (HUS) in Canada and implicated *E. coli* strains of various serotypes that produced a cytotoxin active on Vero cells. Concurrently, investigators from the CDC reported an outbreak of bloody diarrhea (called hemorrhagic colitis) due to *E. coli* of an unusual serotype, O157:H7, that was linked to consumption of fast-food hamburgers in the USA. O'Brien and colleagues showed that such strains produced a phage-encoded Shiga-like toxin that was the same as the verocytotoxin. Studies by Tzipori and colleagues showed that O157:H7 strains produced intestinal attaching and effacing lesions in piglets that were similar to those produced by EPEC strains. O157:H7 and

similar Shiga toxin-producing strains were termed enterohemorrhagic *E. coli* (EHEC) or more broadly, STEC (Shiga toxin-producing *E. coli*) or VTEC (verocytotoxin-producing *E. coli*). EHEC have been responsible for numerous outbreaks of disease in industrialized countries including an outbreak involving more than 8000 victims in Japan in 1996.

Enteroaggregative *E. coli* (EAEC) was first described in the Kaper lab in the 1980s. The pathotype was first recognized by its distinctive auto-aggregating phenotype in the HEp-2 adherence assay, and this phenotype was associated with diarrheal disease in some early studies in India and Chile. Subsequent work over many years has implicated the organism as a cause of endemic diarrhea, traveler's diarrhea, and possibly persistent diarrhea and growth faltering. Although pathogenesis studies have described a large regulon of genes under the control of AraC/XylS family regulator AggR, the extreme mosaicism of the EAEC pan-genome has impeded efforts to generate a clear understanding of its role as a global pathogen. Future studies will need to yield a better definition of what gene complement comprises a true EAEC enteric pathogen.

Additional classes, or pathotypes, of diarrheagenic *E. coli* have been described. Strains that adhere to HEp-2 cells in a diffuse adherence pattern have been termed diffusely adherent *E. coli* (DAEC) and reported to be associated with diarrheal disease in some epidemiological studies but unassociated with disease in other studies. Adherent Invasive *E. coli* (AIEC) have been associated with Crohn's disease, but no unique virulence factors have yet been described for this pathotype. Host genetics, microflora, and chronic inflammation are hypothesized to be involved in disease associated with AIEC.

Enteroinvasive *E. coli* (EIEC) are taxonomically indistinguishable from *Shigella* at the species level, but owing to the clinical significance of *Shigella*, a nomenclature distinction is still maintained based on a few minor biochemical tests. Four *Shigella* species and EIEC cause varying degrees of dysentery, but in most cases, EIEC causes watery diarrhea that is indistinguishable from that due to other diarrheagenic *E. coli*. However, an important distinction is made with *S. dysenteriae* 1, which produces Shiga toxin, unlike other shigellae and EIEC.

EIEC and shigellae invade the intestinal epithelial cells by virtue of a plasmid-encoded type III secretion system and associated effector proteins, which allow the organism to counteract initial host immune responses, mediate invasions, escape the phagolysosome, rearrange host cytoskeleton, destabilize tight junctions, and spread laterally among epithelial cells via actin-based motility.

In 2011, a large multi-country outbreak of hemorrhagic colitis and hemolytic-uremic syndrome was caused by a strain of the unusual STEC serotype O104:H4. Molecular studies of the strain revealed that it was a typical EAEC strain indigenous to Africa but which had become lysogenized with a Shiga toxin-encoding phage. Retrospective analyses of strain collections revealed that this organism had been previously implicated in human infections, but had not been recognized as a lysogenized EAEC. Although the strain has not emerged as a global problem, it points up the remarkable plasticity of the *E. coli* genome and suggests that the complete story of *E. coli* epidemiology has yet to be written.

While the great majority of pathogenic *E. coli* strains have been associated with intestinal disease, *E. coli* also cause disease outside the intestinal tract, and such extraintestinal *E. coli* have been called ExPEC. ExPEC is the major cause of community-acquired urinary tract infections (UTI) and is the second most common cause of neonatal meningitis. It is also a leading cause of adult bacteremia. In animals, avian pathogenic *E. coli* is an important cause of respiratory infections, pericarditis, and septicemia in poultry. In extraintestinal infections, the distinction between pathogenic and nonpathogenic *E. coli* strains is not as clear as it is with diarrheagenic pathotypes, since in the appropriate circumstances nearly any *E. coli* strain may gain access to the bloodstream or the urinary tract. In addition to elucidating the pathogenesis of urinary tract infections, the study of uropathogenic *E. coli* (UPEC) has produced several paradigms of bacterial pathogenesis. The first demonstration of molecular Koch's postulates was reported with the cloning and mutation of hemolysin produced by UPEC. The concept of pathogenicity islands was first reported for UPEC, and classic studies of chaperone-usher assembly of fimbriae were performed with this pathotype. The determination of the genome sequence of UPEC strain CFT073 revealed the mosaic structure of pathogenic *E. coli* with only 39 % of predicted proteins shared by *E. coli* K-12, O157:H7, and UPEC.

Although the broad categories of pathogenic *E. coli* have provided a useful framework to guide investigations, the sheer diversity of virulence factors and the substantial variation within each pathotype greatly complicates the establishment of "hard and fast" rules about this species. A sampling of pathogenic *E. coli* virulence factor activities includes ADP ribosylation of Gs to activate adenylate cyclase and ion secretion, depurination of 28S rRNA to inhibit protein synthesis, DNase I activity to block mitosis in the G2/M phase, disruption of mitochondrial membrane potential, activation of guanylate cyclase resulting in ion secretion, activation of Cdc42 and Rac thereby modulating actin cytoskeleton structure, and microtubule destabilization. These virulence factors are frequently encoded on mobile genetic elements such as plasmids, phage, transposons, and pathogenicity islands. Further details and primary literature citations on these virulence factors and the history of the discovery and recognition of the various *E. coli* pathotypes can be found in several comprehensive reviews (see different chapters in the book).

The breadth of activities of virulence factors and the substantial genetic variation demonstrated by genome sequence studies greatly complicates the task of determining which strains of *E. coli* may be pathogens and which are non-pathogens. The ongoing evolution of pathogenic *E. coli*, as demonstrated by the 2011 O104:H4 outbreak in Europe, makes it very difficult to have a static definition of pathotypes. Future research efforts should more fully characterize the role of coinfections and host factors to gain a more comprehensive picture of disease due to pathogenic *E. coli*.

In this book, *Escherichia coli in the Americas*, members of the Latin American Coalition for *E. coli* Research (LACER) provide a comprehensive review of the different categories of *E. coli* including aspects such as virulence mechanisms, environmental niche, host reservoir, disease outcomes, diagnosis, treatment, and vaccine development. Over the past 50 years, several landmark studies in Latin America have

yielded important insights into pathogenic *E. coli* such as the classic epidemiological studies in Brazil by Trabulsi and colleagues and the studies in Chile that identified EAEC as an important diarrheal pathogen. The lessons learned in Latin America have widespread significance for the study of *E. coli* throughout the world, and the information contained in this volume will be of value for a wide audience, from students to experts, from molecular biologist to epidemiologist.

James B. Kaper, PhD
University of Maryland Medical School
Baltimore, MD, USA

James P. Nataro, MD, PhD
University of Virginia School of Medicine
Charlottesville, VA, USA
June 30, 2016

Key Review References

Friedmann HC (2014) Escherich and Escherichia. In: EcoSal Plus. 6(1). doi:10.1128/ecosalplus.ESP-0025-2013

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201

Kaper JB, Nataro JP, Mobley HLT (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140

Kaper JB, O'Brien AD (2014) Overview and historical perspectives. In: Sperandio V, Hovde CJ (eds) Enterohemorrhagic and other shiga toxin-producing *Escherichia coli*. American Society for Microbiology Press, Washington, DC. Microbiol Spectr. 2(6). doi: 10.1128/microbiolspec.EHEC-0028-2014

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent advances in understanding enteric pathogenic *Escherichia coli.* Clin Microbiol Rev 26:822–880

Preface

In an interconnected and interdependent world, an outbreak caused by an infectious agent in a country is of significant concern because it could result in a sanitary emergency affecting other countries. Globalization has greatly impacted the American continent at different levels, including opening markets and aiding nations to sell their products outside of the country, increasing the real-time communication abilities and allowing an emphasis on international cooperation, as well as many other financial, cultural, and touristic benefits. However, this increased connectivity also potentiates the risk of dissemination for emerging or reemerging infectious diseases. In the case of *Escherichia coli*, a bacterium that is considered a benign as well as a pathogenic organism, globalization has created a scenario in which a pathogenic *E. coli* causes regional outbreaks that can quickly disseminate to other countries. If such bacterial strains are resistant to one or more antibiotics, this becomes a global health threat and alerts have to be in place to notify the authorities and the health providers about such incidents. Globalization in the food chain supply and the distribution of food products to different markets and populations increases the possibility of a rapid spread of an infection caused by *E. coli* and other pathogenic organisms. So there is need for rapid response and effort of the scientific community to identify, diagnose, and understand the pathogenic *E. coli* responsible for the disease.

As such, the Latin American Coalition for *E. coli* Research (LACER) was created in 2009, to promote and expand research efforts in the American continent, to support and expand the best science, to prepare the next generation of scientist-physicians and research investigators, and to work together with the community to translate scientific findings into products improving the well-being of the population. In 2016, the LACER group consist of a multidisciplinary network of more than 60 international research groups working on different aspects of pathogenic *E. coli*, including but not restricted to epidemiology, pathogenesis, vaccine and therapeutic design and testing, public health, surveillance, and clinical identification and treatment.

One major goal of the educational mission of LACER is to advance our understanding about this pathogen and disseminate such knowledge to the region and to the world. Since its inception, some of the educational activities of LACER have included workshops, symposiums, and minicourses for students, scientists, professors, and the

public in general, in different Latin American countries and the USA. In 2010, the members of LACER decided to produce a book entitled *Pathogenic Escherichia coli in Latin America*, which allowed leading investigators in the Latin American region to discuss the mechanisms of *E. coli* pathogenesis as well as the methods of diagnosis, clinical management, host immune responses, animal reservoirs, and epidemiology. In addition, the book discussed epidemiological and public health issues regarding pathogenic *E. coli* in representative Latin American countries.

As the LACER science grew strong and contributions started getting recognized in different forums and social media platforms, the membership, which has expanded significantly, decided to produce a new book in which broader aspects of the pathogens' lifestyles and the diseases they produce were discussed. The current book entitled *Escherichia coli in the Americas* is a compilation of chapters by a large number of *E. coli* experts in Latin America, the USA, and Canada. The book is divided into three major areas: The first includes chapters describing individual pathogenic *E. coli* strains and their different virulence mechanisms used to cause disease. The second includes common mechanisms used by this bacterium to interact with animal or plant hosts (human, animals, and food products) and to resist killing by antibiotics, etc. The third includes chapters devoted to the diagnostics, therapeutic interventions, and vaccine design.

Through the years, LACER members have created a special bond, and this group has become more than just some people working together. It has resulted in unique combination of talent, expertise, and collaborative attitudes that makes the group stronger together than apart. Everyone involved in collaborative research at LACER has a role to play in building our understanding about the always evolving *E. coli*, and advancing technologies and methodologies to diagnose, treat, and prevent such infections have a shared goal of protecting the public health.

Galveston, TX, USA Alfredo G. Torres, MS, PhD

Contents

Contributors

Margarita M.P. Arenas-Hernández Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

Glen D. Armstrong Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada

Adriana B. Bentancor Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Microbiología, CABA, Argentina

Victoria Brusa Facultad de Ciencias Veterinarias, IGEVET—Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET LA PLATA), Universidad Nacional de La Plata, La Plata, Argentina

Angel Cataldi Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina

Nayaret L. Chamorro Universidad de Chile, Santiago, Chile

Isabel Chinen Servicio Fisiopatogenia, Departamento de Bacteriología, Instituto Nacional de Enfermedades Infecciosas "Carlos G. Malbrán", Buenos Aires, Argentina

Roberto J. Cieza Department of Microbiology, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD, USA

Wanderley Dias de Silveira Bacterial Molecular Biology Laboratory, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), São Paulo, Brazil

Waldir P. Elias Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil

Teresa Estrada-Garcia Department of Molecular Microbiology, CINVESTAV-IPN, Mexico City, Mexico

Analía I. Etcheverría CIVETAN, UNCPBA, CICPBA, CONICET, Facultad de Ciencias Veterinarias, Laboratorio de Inmunoquímica y Biotecnología, Tandil, Argentina

Ulysses Fagundes-Neto Departamento de Pediatria, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil

Mauricio J. Farfán Departamento de Pediatría, Facultad de Medicina, Universidad de Chile, Hospital Dr. Luis Calvo Mackenna, Santiago, Chile

José Flores-Figueroa JM Research SC, Cuernavaca, Morelos, Mexico

Lucía Galli Facultad de Ciencias Veterinarias, IGEVET—Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET LA PLATA), Universidad Nacional de La Plata, La Plata, Argentina

Jorge A. Girón Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

University of Virginia, Puebla and Charlottesville, Mexico, VA, USA

Tânia A.T. Gomes Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo—Escola Paulista de Medicina, São Paulo, Brazil

Oscar G. Gómez-Duarte Division of Pediatric Infectious Diseases, Vanderbilt University School of Medicine, Nashville, TN, USA

Beatriz E.C. Guth Department of Microbiology, Immunology, and Parasitology, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil

Rodrigo T. Hernandes Departamento de Microbiologia e Imunologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu, São Paulo, Brazil

Alejandra Krüger CIVETAN, UNCPBA, CICPBA, CONICET, Facultad de Ciencias Veterinarias, Laboratorio de Inmunoquímica y Biotecnología, Tandil, Argentina

Mariano Larzábal Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina

Janaína L. Leite Bacterial Molecular Biology Laboratory, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), São Paulo, Brazil

Gerardo A. Leotta Facultad de Ciencias Veterinarias, IGEVET—Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET LA PLATA), Universidad Nacional de La Plata, La Plata, Argentina

Paula M.A. Lucchesi CIVETAN, UNCPBA, CICPBA, CONICET, Facultad de Ciencias Veterinarias, Laboratorio de Inmunoquímica y Biotecnología, Tandil, Argentina

Daniela Luz Laboratório de Bacteriologia, Instituto Butantan, São Paulo, SP, Brazil

Renato P. Maluta Bacterial Molecular Biology Laboratory, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), São Paulo, Brazil

Claudia F. Martinez de la Peña Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

Mario Meza-Segura Department of Molecular Microbiology, CINVESTAV-IPN, Mexico City, Mexico

Elizabeth Miliwebsky Servicio Fisiopatogenia, Departamento de Bacteriología, Instituto Nacional de Enfermedades Infecciosas "Carlos G. Malbrán", Buenos Aires, Argentina

Fernando Navarro-Garcia Department of Cell Biology, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México, DF, Mexico

Theresa J. Ochoa Departamento de Pediatría, Instituto de Medicina Tropical, Universidad Peruana Cayetano Heredia, Lima, Peru

Center for Infectious Diseases, School of Public Health, University of Texas Health Science Center, Houston, TX, USA

Juan M. Oteiza Laboratorio de Microbiología de los Alimentos, Centro de Investigación y Asistencia Técnica a la Industria (CIATI AC)-CONICET, Centenario, Argentina

Nora L. Padola CIVETAN, UNCPBA, CICPBA, CONICET, Facultad de Ciencias Veterinarias, Laboratorio de Inmunoquímica y Biotecnología, Tandil, Argentina

Marina S. Palermo Instituto de Medicina Experimental (IMEX)-CONICET—Academia Nacional de Medicina, Buenos Aires, Argentina

Mercedes Paredes-Paredes JM Research SC, Cuernavaca, Morelos, Mexico

Roxane M.F. Piazza Laboratório de Bacteriologia, Instituto Butantan, São Paulo, SP, Brazil

Marta Rivas Servicio Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas—ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina

Ricardo Rodríguez Instituto Nacional de Tecnología Agropecuaria (INTA), Rafaela, Argentina

Thaís C.G. Rojas Bacterial Molecular Biology Laboratory, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), São Paulo, Brazil

Fernando Ruiz-Perez Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, VA, USA

Isabel C.A. Scaletsky Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil

Felipe Schelotto Departamento de Bacteriología y Virología, Facultad de Medicina, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay

Marcelo Signorini CONICET—Estación Experimental Agropecuaria INTA, Rafaela, Argentina

Gustavo Varela Departamento de Bacteriología y Virología, Facultad de Medicina, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay

Roberto M. Vidal Universidad de Chile, Santiago, Chile

Mônica A.M. Vieira Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo—Escola Paulista de Medicina, São Paulo, Brazil

Denise Yamamoto Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo—Escola Paulista de Medicina, São Paulo, Brazil

Chapter 1
Enterotoxigenic *Escherichia coli*

Roberto M. Vidal, Nayaret L. Chamorro, and Jorge A. Girón

Summary Pediatric diarrheal diseases continue to represent a significant health burden in areas of the world with poor sanitation. Hundreds of thousands of deaths, mostly in young children occur each year due to severe acute gastrointestinal disease caused by diverse enteric pathogens. Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of childhood gut illness and death in endemic areas. While the epidemiology of ETEC infections is known for some regions of North, Central, and South American countries, the actual impact in most of the continent is unknown. Despite much research efforts of many investigators, safe and effective vaccines against diarrheal disease caused by ETEC are not yet available. The major challenges in developing such vaccines are the poor immunogenicity of the heat-stable enterotoxin produced by the majority of strains and the array of antigenically diverse colonization factors (CFs) that mediate gut colonization; however, while inducing protective antibodies, they only protect against homologous strains. The use of new multi-epitope fusion antigens consisting of chimeric CFs-toxin fusions lacking toxicity has shown important and promising immunogenicity and protection. We review here the current knowledge on ETEC, its epidemiology in the Americas, and the most important vaccine strategies available.

R.M. Vidal (✉) • N.L. Chamorro
Universidad de Chile, Santiago, Chile
e-mail: rvidal@med.uchile.cl; nayarethalseide@gmail.com

J.A. Girón
Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

University of Virginia, Puebla and Charlottesville, Mexico, VA, USA
e-mail: jagiron@yahoo.com

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_1

1 Diarrheagenic *Escherichia coli*

In the 1940s, it was established that some *E. coli* strains caused intestinal infections and so were designated as enteropathogenic *E. coli* to distinguish them from fecal commensal strains. *E. coli* is a Gram negative, facultative anaerobic bacterium that lives in the human gastrointestinal tract as a member of the gut microbiota (Nataro and Kaper 1998; Dubreuil 2012). *E. coli* is a beneficial organism that protects the epithelium of other harmful bacteria by producing an acidic niche through the metabolism of nutrients, provides the host a source of vitamins B and K, and constantly activates the immune system. However, some strains have acquired mobile genetic elements (e.g., plasmids, pathogenicity islands, transposons, bacteriophages) that code for a myriad of virulence factors that allow bacteria to cause a variety of diseases in healthy individuals, including watery diarrhea, dysentery, sepsis and meningitis, the hemolytic uremic syndrome, and urinary tract infections (Kaper et al. 2004). As new information of novel virulence determinants and serotypes identified in epidemic strains became available, it was then possible to start separating diarrheagenic *E. coli* (DEC) strains into different classes. Currently, based on the presence of defined virulence factors, their epidemiology, and clinical manifestations of the disease DEC strains are classified into six pathogroups: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) or Shiga-toxigenic *E. coli* (STEC), diffuse-adhering *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC) (Nataro and Kaper 1998; Qadri et al. 2005; Dubreuil 2012). The plasticity of the *E. coli* genomes and the ability of these organisms to mobilize and acquire foreign genetic elements allows the emergence of new epidemic strains, sometimes hybrid strains, with hyper-virulent attributes. This was exemplified by the epidemics of hemolytic uremic syndrome occurred in Northern European countries in the summer of 2011 by an EAEC O104:H4 carrying the Shiga toxin genes (Rasko et al. 2011).

2 Global Significance of ETEC Infections

It is estimated that about 280 million cases of diarrhea occur annually in the world, producing approximately 400,000 deaths, the majority of them in young children (Steffen et al. 2005). For many decades, ETEC has led, along with *Shigella*, *Salmonella*, and Rotavirus, the list of the most frequent diarrheal microorganisms in endemic areas worldwide. In the realm of infectious diarrheal diseases, ETEC is responsible for the highest attack rates of morbidity and lethality mainly in children under 5 years of age, living under poor sanitation conditions in the developing world. Recent data provided by the Global Enteric Multi-Center Study (GEMS) showed that ETEC is one of the four main etiologic agents of moderate-to-severe diarrhea in regions of sub-Saharan Africa and South Asia (Kotloff et al. 2013). ETEC is also responsible for diarrhea in travelers ("the Moctezuma's revenge") and military personnel deployed to endemic areas (Diemert 2006; Rodas et al. 2011a).

Since the early 1970s, ETEC infections have been monitored in many countries in the Americas, Southeast Asia, and the Indian subcontinent, particularly in those countries visited by travelers and military personnel who are easy targets of ETEC. The interest for studying the epidemiology of ETEC infections appeared to have dimmed or perhaps shifted to other pathogens in the 1990s, particularly due to the emergence of other DECs such as EPEC, STEC, and more recently EAEC. The advent of more sophisticated molecular techniques accelerated the study of the mechanisms of virulence of these *E. coli* pathogroups, gathering highlights and shifted the interests of many researchers to these pathogens. Nevertheless, new and old die-hard *E. coli*/ETEC researchers continued to study the prevalence of ETEC infections among diarrheal cases in several countries of the American continent and other regions of the world. While in some geographic areas ETEC has been displaced by other DEC, such as EPEC or EAEC, it is clear that children continue to die due to watery diarrhea-causing ETEC. Much has been learned from ETEC research in the last 45 years or so however, children continue to die from ETEC infections due to the lack of safe and effective vaccines against the diarrheal disease caused by this important pathogen.

ETEC is a health burden associated mainly with poverty, and the lack of sanitation, potable water, and sewage treatment in developing countries. The consumption of contaminated food and water, and possibly person-to-person contact account for the transmission and elevated number of diarrheal cases. The ability of the organism to survive at room temperature for extended periods of time in cooked food, raw vegetables, and in drinking and nondrinking water is an important factor in the prevalence of this organism in the communities with poor sanitation.

The bacteria are transmitted by ingestion of contaminated food and water (Curtis et al. 2000) with an infective dose is 10^6–10^{10} colony-forming units (CFU) (Nataro and Kaper 1998). Within a period of 14–50 h after ingestion, these bacteria colonize the epithelial mucosa of the small intestine, producing secretory diarrhea without obvious signs of destroying or invading the epithelium or causing inflammation. In addition to diarrhea, some patients may manifest other symptoms such as headache, fever, nausea, and vomiting. The symptoms usually disappear within the first 5 days without the need for antibiotic treatment. The lethal cases are almost exclusively associated with children, due to severe dehydration and lack of protective immunity.

3 Pathogenicity Mechanisms

3.1 Toxins and Adhesins

The main feature of the pathogenesis of diarrheal disease caused by ETEC is the successful colonization of the surface of the intestinal mucosa and the hypersecretion of water and electrolytes due to enterotoxic activity. ETEC overcomes the nonspecific immunological barriers present in the digestive tract and once on the

intestinal epithelial surface, the bacteria multiply and secrete two potent enterotoxins, a heat-labile (LT) and a heat-stable (ST) toxins, responsible for secretory diarrhea. Generally, LT+/ST+, ST+, and LT+ strains can be found in nature, of which, ST-producing strains are presumably more virulent than LT-only strains. ST is a short non-immunogenic peptide and has two variants, STh produced by ETEC strains isolated from humans and STp produced by ETEC isolated from humans and pigs (Nataro and Kaper 1998; Dubreuil 2012). ST binds to the guanylyl cyclase receptor in the intestinal surface and stimulates its action, increasing intracellular levels of cyclic guanosine monophosphate (cGMP). This leads to the activation and opening of the regulator of the transmembrane conductance of cystic fibrosis (CFTR), which permits the exit of chloride ions and water to the intestinal lumen (Dubreuil 2012).

LT is a holotoxin of the AB5 type (1 A and 5B subunits) (Spangler 1992). The B subunit forms a pentameric structure and interacts with the mono-ganglioside GM_1 receptor on intestinal epithelial cells, which facilitates the internalization of the toxic A subunit-pentamer B complex in cell membrane lipid rafts. The complex is then transported to the cytosol by retrograde transport through the Golgi apparatus and the endoplasmic reticulum. Following, the subunit A which possesses ADP-ribosyltransferase activity transfers an ADP-ribosyl residue from an NAD^+ molecule to the α subunit of protein G, which regulates the activity of the adenylate cyclase enzyme (Mudrak and Kuehn 2010). This leads to an increase in the intracellular level of cyclic adenosine monophosphate (cAMP), stimulating cell kinases, which phosphorylate and activate the CFTR channel, liberating chloride ions and water to the intestinal lumen. The increase of intracellular cAMP activates protein kinase A (PKA), which affects sodium absorption by means of the interchanger 3 of Na^+/H^+ (NHE3), allowing subsequent liberation to the intestinal lumen (Dubreuil 2012). The massive loss of electrolytes and water into the intestinal lumen produced both by ST and LT is what is finally manifested as watery diarrhea in the host (Dubreuil 2012). Notably, the B subunit is considered to be a potent mucosal adjuvant. Both LT-A and LT-B elicit immuno-protective antibodies in humans, following natural ETEC infections, and in animals after experimental challenge. Thus, it is a suitable immunogenic candidate in ETEC vaccines. The ST, on the contrary, is poorly immunogenic, and this is in part the reason why vaccines against ST-producing ETEC have not been successful.

3.2 Nonclassical Toxins, Mucinases, and Metalloproteases

Several other potential virulence factors (e.g., EAST-1, EatA, LeoA, YghJ) have been found in ETEC strains although none of them has been associated with disease in humans (Gonzales et al. 2013b; Fleckenstein et al. 2014). A third toxin initially described in EAEC namely the EAEC heat-stable toxin (EAST-1) was also reported in some ETEC and other DEC strains. The *ast* gene encoding EAST-1 is found associated with mobile elements, which explains its distribution among the several DEC. Similar to ST, EAST-1

produces an increase in intracellular levels of cGMP, which presumably may produce an additive effect in the watery diarrhea process (Fleckenstein and Sheikh 2014).

Some ETEC strains produce a proteinase called ETEC autotransporter (EatA), a member of the family of self-exported proteins referred to as serine protease autotransporters of the *Enterobacteriaceae* (SPATE). EatA is a mucinase that degrades MUC2, the dominant mucin glycoprotein in the intestinal mucus layer, and it is also capable of degrading EtpA, an ETEC two partner adhesin. EtpA is a novel flagella-associated tip adhesin of some ETEC strains, which facilitates toxin delivery (Roy et al. 2011; Kumar et al. 2014; Fleckenstein et al. 2014). EatA was found in 50 and 70 % of ETEC strains in Peru and Chile, respectively (Rivera et al. 2013; Del Canto et al. 2011). Vaccination of mice with EatA showed protection against experimental intestinal infection with an ETEC homologous strain (Kumar et al. 2014).

A cytoplasmic protein with GTPase activity was found to influence positively the secretion of LT hence named Labile Enterotoxin Output (LeoA) (Fleckenstein et al. 2000). The gene coding for LeoA is present in a 46-kb pathogenicity island that also carries the *tia* gene, which was found in 6 % of children ETEC isolates (Fleckenstein et al. 2000; Gonzales et al. 2013b). The low frequency of *leoA* among ETEC strains argues against a relevant role in pathogenesis.

The cytolysin A (ClyA) is a pore-forming protein initially described in *E. coli* K-12. The *clyA* (also called *sheA*) gene is present in most pathogenic *E. coli*, including ETEC, and it was suggested that ClyA could contribute to virulence of some pathogroups. ClyA recognizes membrane-associated cholesterol as a receptor (Ludwig et al. 2004). A survey of *clyA* among ETEC strains showed that more than 90 % of the isolates contained this gene (Turner et al. 2006).

A metalloprotease encoded by the chromosomal *yghJ* gene was found widely distributed in DEC and *Vibrio cholerae*. YghJ was reported to influence the ability of ETEC to colonize the small intestine targeting degradation of MUC2 and MUC3 intestinal mucins (Luo et al. 2014). More investigation is required to elucidate the role of these new potential virulence factors in the pathogenic scheme of ETEC.

3.3 Adherence Factors

The interaction of ETEC strains with and colonization of the epithelial mucosa in the small bowel is mediated by at least 26 fimbrial adhesive structures collectively called “coli surface antigens” (CS1-CS26) (Madhavan and Sakellaris 2015). Upon the discovery of the first two ETEC fimbrial types in the late 1970s by Dolores Evans, a Mexican researcher, they were originally called “colonization factor antigens” (CFAs) (e.g., CFA/I and CFA/II) (Evans et al. 1975). The CFA/III was discovered by T. Honda in ETEC serogroup O25 isolated in Japan (Honda et al. 1984). The CFA/IV was found in ETEC isolates from traveler’s diarrhea by British investigators (Thomas et al. 1982; Thomas et al. 1985). It was later on realized that CFA/II was composed of CS1, CS2, and CS3 and CFA/IV of CS4, CS5, and CS6, in different permutations (Smyth 1982; Thomas et al. 1985; Levine et al. 1984). More

curiously than interesting, the term CS was coined by ETEC researcher Cyril Smyth (Smyth 1982). Currently, only CFA/I and CFA/III conserve the original designation in recent reviews of ETEC adherence factors, while the remaining 24 CFs are referred to as CSs. For historical reasons and to honor the researcher who first described and named the first ETEC colonization factors in this chapter, we will refer to all these fimbrial antigens collectively as CFs.

The genetic determinants dictating the production of CFs are generally harbored in virulence plasmids although some are located in the ETEC chromosome (Madhavan and Sakellaris 2015; Qadri et al. 2005). The stalk of the fimbrial CF structure is generally composed of thousands of copies of one major fimbrial subunit. For a handful of known CFs, a minor pilin subunit, which is located at the tip of the filament, is responsible for receptor recognition on host cells. In the case of CS3 and CS6, two major protein subunits (CS3A and CS3B, and CS6A and CS6B) comprising the extracellular product have been found. The biochemical nature and spatial distribution of the major and minor subunits determine fimbrial morphology (Del Canto et al. 2012; Madhavan and Sakellaris 2015). An ETEC strain may express one or more CFs in different combinations and their distribution varies according to the geographic region of the world (Qadri et al. 2005). The most frequent CFs in ETEC strains around the world are CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17, and CS21. Nevertheless, over 50 % of the epidemic strains analyzed worldwide lack any of the known CFs (Giron et al. 1995; Qadri et al. 2005; Del Canto et al. 2011; Rodas et al. 2011b; Del Canto et al. 2012; Madhavan and Sakellaris 2015). This has been a major hurdle in the development of fimbrial-based vaccines against ETEC because the vaccines tested so far are not protective against heterologous strains producing different or unknown CFs. Thus, more research is needed to identify new CFs in ETEC that are wrongfully called CF negative.

A new highly prevalent CF has been described in ETEC strains. The meningitis-associated temperature-dependent fimbriae (MAT) was reported in septicemic and meningitis-causing *E. coli* strains growing at temperature below 37 °C (Pouttu et al. 2001). Although initially thought to be exclusive of these extra-intestinal *E. coli*, it was later found that MAT are produced at 37 °C by all human and animal pathogenic *E. coli,* including commensal strains. These fimbriae are encoded in the genetic core of all *E. coli*; hence, it was suggested that MAT be renamed *E. coli* common pilus (ECP) to better describe the wide distribution of this pilus among this organism (Rendon et al. 2007). Blackburn et al. and others have shown that the *ecpA* gene is certainly the most prevalent CF in all ETEC (Del Canto et al. 2011; Rivera et al. 2013). ECP mediates bacterial attachment to human, animal, avian, and plant cells through the EcpD tip adhesin and is involved in the development of biofilms by various *E. coli* pathogroups, including ETEC (Rendon et al. 2007; Blackburn et al. 2009; Garnett et al. 2012). The biological relevance of ECP, in the context of ETEC diarrheal disease, remains elusive. However, the fact that ECP is expressed along with other CFs and is highly prevalent among ETEC strains should not been ignored as it is reasonable to assume that this synergy would increase the adhesiveness of the bacteria.

Although CFs are known for over 40 years, the biochemical nature of the intestinal receptors for the majority of them is unknown. Asialo-glycosphingolipids are implicated as receptors for CFA/I-mediated adherence to Caco-2 cells and erythrocytes (Madhavan et al. 2016) and a sulfatide glycosphingolipid (SO3-3Gal-β1Cer) was proposed as the receptor for CS6 (Jansson et al. 2009). In contrast, the receptor binding moiety of some of the fimbriae of animal ETEC (e.g., K88, K99, F17G, F4) have been elucidated and used successfully to achieve protection in pigs or calves (Lonardi et al. 2013); (for further details, see Chap. 15). Finally, the long polar fimbriae genes (Lpf) and their variants initially identified in Shiga toxin-producing *E. coli* also have been described in other diarrheagenic pathogroups of *E. coli* including ETEC strains (Torres et al. 2002; Toma et al. 2006).

3.4 Nonclassical Adhesins

In recent years, a group of non-fimbrial adhesins EtpA, Tia, TibA, TleA, and EaeH referred to as "nonclassical adhesins" was described and characterized mostly in the prototypic strain ETEC H10407A (Fleckenstein et al. 2014). The distribution of the genes encoding these potential adherence factors among ETEC strains varies greatly. EtpA, a high-molecular-weight glycoprotein encoded on the *etpBAC* locus harbored on the virulence plasmid of H10407 was suggested to participate in adherence to cultured intestinal epithelial cells. Later on, it was reported that EtpA is a flagellum tip-associated protein that allows bacterial interaction with cultured epithelial cells and protects mice vaccinated with the glycoprotein or flagella (Roy et al. 2009; Roy et al. 2008). Although this represents a novel mechanism of bacterial adherence, it is not a generalized feature of ETEC strains. The *etpABC* locus was found in about 20 % of ETEC strains from different geographic origins but not in other DEC (Fleckenstein et al. 2006). However, *etpA* was detected in more than 70 % of Chilean ETEC strains studied (Del Canto et al. 2011).

The toxigenic invasion loci A and B encode two proteins, Tia and TibA, which were suggested to be associated with adherence and invasion of cultured epithelial cells by ETEC H10407A (Fleckenstein et al. 1996; Lindenthal and Elsinghorst 1999). TibA is an autotransporter glycoprotein that mediates bacterial attachment to a variety of cultured human epithelial cells, autoaggregation, and biofilm formation (Sherlock et al. 2005; Cote and Mourez 2011). While a series of reports on Tia and TibA implicate them as adhesins/invasins, the invasion and possible damage to the small bowel by ETEC strains in humans is not a feature of the pathology seen in diarrheal cases. Further, the invasion rates of H10407A in cultured epithelial cells are far lower than those reported for intracellular pathogens: *Salmonella enterica* serovars or *Shigella*. It is possible, however, that these proteins play perhaps a marginal role at some stage in the diarrheal process.

Recently, a homolog (97 % identity, 90 % coverage) of the temperature-sensitive hemagglutinin (Tsh) autotransporter protein previously described in avian pathogenic *E. coli* strains was reported in ETEC and called TleA (Tsh-like ETEC)

(Gutierrez et al. 2015). When expressed in a nonadherent *E. coli* K-12 strain HB101, TleA confers Caco-2 cell adherence properties. In addition, TleA showed mucinase activity degrading leukocyte surface-exposed glycoproteins and mucin, suggesting that it may contribute to the colonization of the intestine epithelium and modulation of the immune response (Gutierrez et al. 2015).

Finally, it was suggested that the EaeH protein contributes to bacterial adherence and virulence; however, the *eaeH* gene is not ETEC specific since it is found in other *E. coli* (Sheikh et al. 2014; Luo et al. 2014). While the significance of these "nonclassical virulence factors" in the establishment or progression of ETEC diarrheal disease is not confirmed, it is apparent that some of these determinants might play, in combination with the CFs, some synergistic role in the adhesive properties of some ETEC strains. Comparative adherence studies, particularly in animal models, employing isogenic CF, EtpA, or EaeH mutants would help understand their true contribution to bacterial attachment.

4 Epidemiology of ETEC Diarrheal Disease in Latin America

Several epidemiological studies of diarrheal disease were conducted during the period 1974–1987 in adult populations of students and tourists, as well as Peace Corps volunteers working in Latin American countries, to determine the etiologic causes of infection (Black 1990). In all, ETEC was the bacterial enteropathogen most frequently identified, with a median of 42 % of the diarrhea episodes in travelers; this was greater than those described for Africa (36 %) and Asia (16 %). Additionally, one of the first reports of acute diarrhea in the pediatric population in Latin America was a study that evaluated the impact of oral rehydration in the treatment of ETEC infections as recommended by the World Health Organization (WHO) (McLean et al. 1981; Del Canto et al. 2011). This study was performed in Fortaleza, Brazil from January 1977 to June 1978 and found that ETEC strains were responsible for 27 % of the cases of diarrhea in children age 8–19 months old (McLean et al. 1981).

In the next sections, we will summarize the latest epidemiological studies conducted in America, the continent, with emphasis on the role of ETEC as a health burden protagonist.

4.1 ETEC in Chile

During a study of epidemiological vigilance performed in the 1980s, 103 strains of ETEC were collected from children under 5 years of age, who had diarrhea and lived in a peripheral population in the city of Santiago, Chile (Del Canto et al. 2011). The O somatic antigens were detected by serology and enterotoxins (LT and ST)

and classical and nonclassical adhesins were identified by PCR. Ten and 2 % of the strains harbored known CFs and known classical adhesins, respectively, while 73 % had a combination of CFs and nonclassical adhesins. The most common surface determinants found were EtpA (75 %), CS21 (73 %), CS3 (26 %), CS1 (13 %), and CS2 (13 %). However, in spite of including nonclassical adhesins in the analyses, 16 % of the ETEC strains remained negative for any known adhesin (Del Canto et al. 2011), suggesting the presence of unknown variants. A study conducted during the period 1988–1989 in a low socioeconomic level peri-urban community in Santiago, with access to chlorinated water, described a frequency of ETEC isolation of 12.3 % in a cohort of 340 children (Levine et al. 1993). Interestingly, two subsequent studies performed in the pediatric population of the same semirural zone in the years 2002–2003 (Vidal et al. 2004) and 2004–2005 (Vidal et al. 2005), found isolation frequencies of ETEC of 3.1 % and 1.9 %, respectively. Clearly, the significant reduction in ETEC burden was the result of the introduction of sewage water treatment in the community.

4.2 ETEC in the Highlands of Bolivia

In a retrospective study involving 3943 cases of childhood diarrhea and 1026 children without diarrhea, ETEC and EPEC came second at similar rates (~6 %) after EAEC (11.2 %). Diarrheal disease due to EPEC, ETEC, and EAEC infections peaked in the Bolivian winter months (April–September). All together, these DEC had high levels of antibiotic resistance to tetracycline and sulfamethoxazole-trimethoprim (Gonzales et al. 2013a).

In a 4-year investigation, 299 strains of ETEC isolated from children with diarrhea seen at local hospitals in the regions of La Paz and Albina Patiño in Cochabamba, and from 55 children without diarrhea, were screened for toxin and CF profiles. Climatically and socioeconomically, these are two very different regions of Bolivia. Strains bearing LT or ST only were found 2–3-fold more frequently than LT/ST producers among diarrheic patients. This latter group of strains was most commonly found in children between 2 and 5 years of age while STh+ strains were found among younger children. Interestingly however, the severity of disease was not related to toxin profile. In the 55 children without diarrhea, LT+ ETEC were more frequent than in the sick children, followed by ST+ and LT+/ST+ strains. In agreement with previous epidemiological studies, LT-only ETEC can be found in children without diarrheal symptoms, which has suggested that perhaps LT-only strains are less virulent than ST-only ETEC (Kotloff et al. 2013). However, in this population, depending of the year of study, LT+ ETEC strains were associated as frequently as ST+ ETEC with more cases of diarrhea. The search for CFs among the isolates revealed that CF+ strains were more common in diarrheal samples than in controls. CFA/I followed by CFA/II, CFA/IV, CS12, CS13, CS14, and CS21 were the most common fimbrial genes found in strains from diarrheic patients. Of note, CS17 was found in 2–6 % of the strains in diarrheic patients,

depending on the year of study, but it was as frequent as CS14 in controls. It remains to be determined if the *csb* gene, encoding the CS17 pilin, is related to CS23 recently described in Chilean ETEC strains, which is 97 % identical to CS17 (Del Canto et al. 2012). Given the geographic vicinity of Bolivia and Chile, this notion is not far-fetched. The new virulence factors *clyA*, *eatA*, *tia*, *tibC*, *leoA*, and *east-1* genes were found at varying frequencies in both study groups but were not associated with diarrheal cases. The *east-1* and *tia* genes were associated with LT/ST strains while *eatA* was commonly found associated with ST strains (Gonzales et al. 2013a). Further analysis by Multilocus Sequence Typing (MLST) of LT+ CS17+ ETEC strains revealed two major sequence types, 423 and 443. All strains of the sequence type 423 had a single nucleotide polymorphism in the CS17 operon and were resistant to erythromycin, penicillin, chloramphenicol, and oxacillin (Rodas et al. 2011a).

4.3 ETEC in Colombia

In a handful of survey studies performed in different regions of Colombia, the frequency of several DEC has been addressed. DEC pathogroups were sought by multiplex PCR in 108 diarrheal stool samples from children attending 6 hospitals in Bogota, as well as in 76 food product (meat and vegetables) samples. Sixty-seven samples positive for *E. coli* strains were isolated from stools of children with diarrhea, 16 from meat samples, and 12 from vegetable samples. As expected, various DEC pathogroups were found at different ratios in diarrheic and food samples. Six, 3, 2, 1 of the clinical samples had EPEC, EAEC, ETEC, and STEC, respectively. The combined results of diarrheal and food samples showed that 10 % had any DEC. Eleven percent of the clinical samples had EPEC and 8 % of the food samples had any *E. coli* pathogroup. No information was provided regarding the background *E. coli* pathogroups circulating in healthy children in this community (Rugeles et al. 2010).

A recent study looked for *E. coli* pathogroups in cheese, pasteurized milk, unpasteurized juice, ground beef, and vegetables and found that 8 % of the samples had DEC, among which STEC and ETEC were almost equally present in ground beef (Amezquita-Montes et al. 2015).

A different study of diarrheal diseases performed in the Northern region of Colombia, namely the cities of Cartagena and Sincelejo, involved 267 stool samples from children less than 5 years of age with diarrhea collected during 2007. No healthy controls were examined. Among them, 139 *E. coli* isolates were recovered, 28 from Cartagena, and 111 from Sincelejo. Twenty of the 139 (15 %) *E. coli* isolates amplified any of the virulence genes sought. Overall, 7.5 % of the total diarrheic samples had DEC. ETEC was found in 7 % of the 139 *E. coli* isolates, mainly in children below 2 years old. However, none of the associations of pathogens displayed statistical significance, attributed perhaps to the lack of healthy control group for comparison (Gomez-Duarte et al. 2010).

More recently, a case-control study (349 pairs) conducted to evaluate the association of DEC with diarrheal disease in children younger than 5 years of age, who attended Napoleon Franco Pareja Children's Hospital in Cartagena. A total of 349 pairs were matched; however, the ratio of 1:1 of cases and controls were not paired for any variable. The stool samples from matched controls came from the same hospital and from day-care centers in the vicinity of the hospital. DEC were found in 7.4 % cases and in 3.4 % controls. ETEC was the most common pathogen when comparing cases (4.9 %) versus controls (3 %) although these results do not appear to indicate an association with disease. Most of the ETEC strains isolated from diarrheal cases were ST+ (9 of 17). In contrast, 7/8 control children had LT+ ETEC. The presence of other *E. coli* pathogroups was negligible in this pairing system (Gomez-Duarte et al. 2013).

Forty ETEC strains isolated from diarrheic children and controls originated from the studies described above were assayed for the presence of various CFs and new putative virulence factors. One half of the clinical ETEC strains had LT genes while 32 and 15 % of the isolates were ST+ or LT+/ST+, respectively. Longus gene was detected in 50 % of the clinical ETEC strains in association with CFA/I or other CSs in particular with ST+ ETEC. The CFA/I was present in 32 % of ST-only ETEC. CS5 and CS6 were found in 12 and 20 % of the strains, respectively. New putative virulence factor genes *irp2*, *fyuA*, and *eatA* were present in 33 (82.5 %), 30 (75 %), and 29 (72.5 %) of ETEC isolates, respectively. Sixty percent of the strains had these three genes. In contrast, strains carrying the *tia*-PAI-associated genes were uncommon. Only 1 (2.5 %) strain was positive for the *leoA* gene while *tia* and *tibA* were found in 7 (17.5 %) isolates. Forty-five percent of the strains carried *etpA* and *etpB* virulence plasmid genes. Eighty percent of the ETEC strains were resistant to ampicillin and trimethoprim-sulfamethoxazole. Strains resistant to ampicillin, trimethoprim-sulfamethoxazole, cefazolin, and amoxicillin clavulanate were detected in 67 %, 50 %, 15 %, and 5 % of the isolates, respectively. No resistance to ceftriaxone, ceftazidime, cefepime, ciprofloxacin, and piperacillin/tazobactam was detected among ETEC isolates. Based on multiple locus sequencing typing (MLST), six clonal groups of ETEC clinical isolates were recognized in Northwest Colombia indicating that highly diverse in terms of virulence factors, serotypes, and MSLT types (Guerra et al. 2014).

4.4 ETEC in the Andes

The most recent studies of childhood diarrheal disease in the Andes region of South America come from Peru and Ecuador. While ETEC continues to be responsible for some attacks rates of diarrhea in children, some studies also have found ETEC associated with illness in HIV patients (Garcia et al. 2010). A prospective, passive surveillance cohort diarrhea study of 1129 samples from children 2 to 24 months of age with diarrhea and 744 samples from control children living in the peri-urban communities of Lima, Peru, conducted between September 2006 and July 2008 revealed

the presence of ETEC equally in cases and controls (~5 %). The most prevalent ETEC toxin types in both groups of children were LT producers followed by ST+ and then LT+/ST+. As in other studies, a minority of children had mixed DEC infections. About half of ETEC isolates lacked CF as determined by monoclonal antibody screening. The most common CF found was CS6. In this cohort, a high percentage of strains were resistant to ampicillin (71 %) and co-trimoxazole (61 %) (Rivera et al. 2010).

A retrospective study of the prevalence of DEC in Peru was conducted employing *E. coli* strains isolated from 3284 pediatric patients. The samples came from several communities, hospitals, and a cohort study. Samples of non-diarrheic children were also included. The presence of EAEC, EPEC, ETEC, and DAEC varied from 10, 8, 7, to 5 %, respectively. ETEC has found more frequently among 13–20 months-old children.

Gonzaga et al. reported an outbreak of co-infection with ETEC and norovirus genotype 1 among crew members of a US navy ship in Lima, Peru. While the association of ETEC and norovirus has been reported before it was noted that such outbreak was the first to occur on a navy ship in Peruvian waters docking in Lima (Gonzaga et al. 2011).

In a separate study, the antimicrobial susceptibility and mechanisms of resistance of 205 ETEC isolates from two cohort studies in children 24 months of age in Lima, Peru revealed that ETEC from Peruvian children are often resistant to older, inexpensive antibiotics, while remaining susceptible to ciprofloxacin, cephalosporins, and furazolidone. Fluoroquinolones and azithromycin remain the drugs of choice for ETEC infections in Peru (Medina et al. 2015).

In Northwestern Ecuador, several case-control studies were conducted in 16 communities in the Canton Eloy Alfaro, province of Esmeraldas, between November 2004 and December 2010 to investigate the distribution of ETEC and EIEC across the region. The data show that the source of infection may have been Borbón, the main commercial and population center of the region, from where ETEC and EIEC spread to other communities at different time periods (Bhavnani et al. 2016).

4.5 Brazilian ETEC

The studies reported in the last 3 years on the epidemiology of diarrheal diseases, in particular associated to ETEC infections, are too few to represent such a vast territory. A case-matched control study was conducted in a hospital in the city of Joao Pessoa, State of Paraiba in the Northeast of Brazil, which included 2344 *E. coli* isolates from 290 infants with diarrhea and 290 matched controls. Sixty-four percent of the cases occurred during dry season. Ten percent of the *E. coli* isolates were ETEC, 25 % EAEC, and 9.3 % atypical EPEC. ETEC was also found in 3 % of the matched controls. It is clear from this study that EAEC is highly represented in this geographic region. It is important to note that these DEC were sought employing DNA probes specific for virulence factors of the pathogens, as opposed to using multiplex

PCR, which is currently widely used by many laboratories. It is also important to point out that this method may not be 100 % reliable due to natural minor variations that occur in gene sequences of the factors studied among field strains (Moreno et al. 2010). A study conducted in 23 "quilombola" communities with low socioeconomic status located at 265 km (165 miles) North of the City of Espiritu Santo, Southeast of Brazil, included 590 samples from 141 children with diarrhea (<10 years of age) and from 419 healthy controls. A total of 1943 *E. coli* strains were studied for the presence of DEC virulence factors by multiplex PCR. DEC were found in 253 children below 5 years of age (45.2 %) and among these isolates EAEC 21 %, DAEC 11.6 %, EPEC 9.3 %, and ETEC 2.7 % were found. Most of the ETEC strains were LT+/ST+ (Lozer et al. 2013). As in the Northeast of Brazil, EAEC was the most frequent DEC found while ETEC was the least frequent DEC.

4.6 ETEC Infections Among Travelers and Residents in Central America

Tourists visiting Guatemala and Mexico are common "preys" of ETEC. Acute diarrhea is Guatemala's second-leading cause of child morbidity and mortality, with rates as high as 8–11 episodes/child per year (Cruz et al. 1992). A collection of ETEC (CS6+ and STp+) isolates associated with travelers' diarrhea in Antigua, Guatemala and Cuernavaca, Mexico, were targeted for MLST. Strains with sequence type 398 were commonly found in children living in Antigua and adult travelers to this area. In addition, ETEC strains from travelers clustered within the 278 and 182 MLST types. The peak of diarrheal diseases due to bacterial pathogens in Mexico and Central America normally occurs in the summer months while viral diarrheal infections are prevalent in the winter months. A cohort study of US adult students traveling to Cuernavaca followed during 1 year confirmed this notion and revealed that the attack rate of ETEC infection increased by 7 % for each degree centigrade increase in weekly ambient temperature (Paredes-Paredes et al. 2011). Infections by ST+ ETEC in students staying in Mexico for at least 6 months put them at a greater risk of developing persistent abdominal symptoms (Nair et al. 2014).

The significance of ETEC infections in Guatemalan children was studied by (Torres et al. 2015). The study included children aged 6–36 months, with severe diarrhea seen at the Hospital Roosevelt in the city of Guatemala. In addition, community cases were identified by passive surveillance during two consecutive years in children living in the community of Santa María de Jesús (SMJ), Sacatepéquez. These community children were matched with non-diarrhea controls for age, gender, and residence zone. CFs and toxins were sought by immunoassays. The peak of ETEC infections was the summer rainy months of May–September. The authors found that ETEC accounted for 26 % of severe cases of diarrhea in children requiring hospitalization, 15 % of diarrhea in the community, and 29 % of travelers' diarrhea. However, 10 % of the healthy controls also had ETEC. LT+ ETEC were the most common among acute and persistent cases, followed by ST+ ETEC. Notably,

65 % of the isolates had no detectable known CF, a result that is most likely attributed to the monoclonal antibody screening approach. Nevertheless, the most commonly expressed CFs among community ETEC strains were CS6 (95 %), followed by CS1+CS3 or CS2+CS3 in 7 % and CS4+CS6 or CS5+CS6 in 6 % of the strains. Importantly, this study highlights differences in the strains infecting resident children as compared to ETEC strains infecting visitors.

In Nicaragua, 526 stool samples from 381 children with and 145 without diarrhea living in the city of Leon were screened for the presence of DEC types by multiplex PCR. Although EAEC and EPEC were found in the diarrheic children, only ETEC LT+/ST+ were significantly associated with diarrhea (Reyes et al. 2010).

4.7 ETEC in Mexico

It is no secret that authentic Mexican tacos, of course served in Mexico not in the United States, taste yummier with a coliform-rich green or red hot salsa. The ability of fecal coliforms to withstand the low pH of and to survive and multiply in Mexican salsas at room temperature at mobile street food stands accounts for high rates of diarrheal episodes in the happy and loyal customers. Several studies conducted by Teresa Estrada and others have documented the presence of ETEC, other DEC and enteric pathogens in street food stands (Estrada-Garcia et al. 2009; Patzi-Vargas et al. 2015). More recently, a series of reports conducted in the city of Pachuca, Mexico, relate the significance of the presence of enteric bacteria in vegetables, fruits, juices, and other eatable agricultural products. These studies reveal that tomatoes, alfalfa sprouts, jalapeno and serrano papers can host DEC. ETEC was detected in 12 % of serrano and 2 % of jalapeno peppers, and in 2–3 % of tomatoes, alfalfa sprouts, and fresh raw beetroot juice available at local public markets in Pachuca (Gomez-Aldapa et al. 2013; Gomez-Aldapa et al. 2014a; Rangel-Vargas et al. 2015). A different study showed that ETEC, EIEC, EPEC, and STEC display similar growth patterns on jalapeno or serrano peppers at 25 or 3 °C although growth after 12 days of incubation was inhibited much faster at 3 °C (Gomez-Aldapa et al. 2014b).

Ready-to-eat cooked vegetable salads sold at a local restaurant in Pachuca were screened for *Salmonella* and DEC by multiplex PCR and bacteriological techniques (Bautista-De Leon et al. 2013). EPEC, STEC non-O157:H7, and ETEC (ST+) were found in only 1.4 % of the samples screened. STEC non-O157 and ETEC strains have been identified from raw foods in Mexico such as vegetable salads and carrot juice (Castro-Rosas et al. 2012; Torres-Vitela et al. 2013).

In Mexico City, fecal samples from 1000 patients with acute diarrhea who attended various hospitals were analyzed for the presence of enteric pathogens. ETEC was found in 54 samples. These ETEC strains were multidrug resistant. Eighty percent of them were resistant to rifaximin, 30 % to ampicillin, and 50–90 % to fosfomycin, trimethoprim-sulfamethoxazole, neomycin, furazolidone, chloramphenicol, and ciprofloxacin (Novoa-Farias et al. 2016). In a different study, ETEC isolates showed increased resistance over the years to

ciprofloxacin and levofloxacin, compared to isolates in Central America. The rising minimal inhibitory concentration (MICs) of ETEC and other DEC justify the need for continuous surveillance of susceptibility patterns worldwide and geographical-specific recommendations on the therapy of diarrheal diseases (Ouyang-Latimer et al. 2011).

The prevalence of DEC was investigated in diarrheic children hospitalized in the Southeast Yucatan peninsula. While DEC surpassed *Salmonella* and *Shigella* in terms of frequency among the cases, the isolation of individual *E. coli* pathogroups ranged in descending order DAEC>EAEC>EPEC>mixed DEC> ETEC. Ten percent of the cases were infected with ETEC strains which also harbored the so-called supplementary virulence genes, generally present in EAEC strains, such as *aap* (dispersin), *aatA* (translocator), *ast* (EAST-1), *pet* (plasmid-encoded toxin), and *cdt* (cytolethal distending toxin). The rationale for searching these genes in ETEC or DEC is not clear since not all of these genes are true virulence factors, and their role in pathogens other than EAEC is largely unknown. Nevertheless, as expected these genes were present in 93 % of EAEC strains and at different percentages in the other DEC categories. One-third of ETEC strains harbored *ast* (30 %) and *aap* (3 %) (Patzi-Vargas et al. 2015).

The presence of Longus, a type IV pilus of ETEC (also known as CS21) (Giron et al. 1994), was recently revisited in strains previously isolated from 5-year-old children in Bangladesh and Mexico. One quarter of the strains carried the Longus major pilus gene *lngA*, but the LngA protein was only detected in 50 % of these (Cruz-Cordova et al. 2014). In previous studies, the frequency of *lngA*+ in Bangladeshi and Mexican ETEC strains was 6.5 % and 36 %, respectively (Qadri et al. 2000; Gutierrez-Cazarez et al. 2000). It is apparent that over time there is a shift and turnover of ETEC strains in these human populations.

The biochemical and genetic diversity of ETEC isolated from US subjects studying in the cities of Cuernavaca or Guadalajara, Mexico during the summer months was studied in a total of 166 strains. Most of the strains were ST+ followed by LT+/ST+ and lastly LT+. Twenty-four clonal groups were identified by Random Amplified Polymorphic DNA RAPD analysis, which indicates extensive genetic diversity among ETEC strains that persist in this particular location (Ouyang-Latimer et al. 2010).

5 Current Status of the Development of Vaccines Against ETEC

The development of an ETEC vaccine is a long needed and important primary prevention strategy against diarrheal disease particularly for children who live in endemic regions and travelers. Despite much research efforts of many investigators, safe and effective vaccines against ETEC diarrheal disease are not available (Fleckenstein et al. 2014; Madhavan and Sakellaris 2015). The major challenges in developing such vaccine are the rich repertoire of CF antigenic mosaics present in

ETEC and the poor immunogenicity of ST, which is a feature of the majority of virulent strains. The ETEC vaccines developed in the past achieved some protection against homologous strains but not heterologous strains. An array of vaccine formulations, including formalin-killed organisms, attenuated *E. coli* and non-*E. coli* strains used as vectors expressing the various CFs, pili-derived peptides, and pili-based vaccines comprising most prevalent CFs combined with toxoid forms of ST and LT has been tested for induction of anti-adherence and anti-toxicity antibody response in animal models and human volunteers (Giron et al. 1995; Barry et al. 2006; Norton et al. 2015; Sincock et al. 2016). The use of new multi-epitope fusion antigens consisting of chimeric CFs-toxin fusions lacking toxicity, promises to achieve important immunogenicity and protection (Ruan et al. 2015; Rausch et al. 2016; Zhang and Sack 2015).

So far, the only ETEC vaccine available is one originally designed to prevent cholera, which contains the recombinant B subunit of the cholera toxin (rCTB). This preparation is trademarked under the name Dukoral® and provides 50–70 % protection against diarrhea caused by ETEC strains that produce LT only and LT/ST. This cross-protection is attributed to the striking structural, functional, and antigenic similarities that exist between CT and LT (Nataro and Kaper 1998; Sanchez and Holmgren 2005). Ideally, an ETEC vaccine should contain a toxoid form of LT, an immunogenic and nontoxic form of ST, and a cocktail of the most prevalent CFs. This vaccine should be a one-dose oral vaccine and achieve long-term protection against heterologous ETEC strains to attain wider coverage, and to protect young children during the first 2–3 years of life. However, only a handful of the most frequent CFs found worldwide has been evaluated as potential vaccines (Sanchez and Holmgren 2005). The demonstrated relatedness between all nine fimbrial types of the Class 5 family composed of CFA/I, CS1, CS2, CS4, CS5, CS14, CS17, CS19, and PCF071 shall help in the formulation of a common antigen that elicits protective antibodies against all these CFs. Of high importance in attaining an ETEC vaccine is the search for new CFs in the so-called CF-negative ETECs. Much has been learned from these vaccination experiences in the last 45 years; however, children continue to die mainly from ETEC and EPEC infections due to the lack of protective vaccines.

For a current status and detailed analysis of ETEC vaccines in early and advanced stages of clinical development and in the preclinical stage, we refer the reader to the review by (O'Ryan et al. 2015). Novelties published in the last 2 years associated with new vaccine studies, and new antigens are described in the Table 1.1.

6 Concluding Remarks

The epidemiological studies of gastrointestinal infections conducted in Latin America in recent years have unraveled the most important causes of infantile diarrheal diseases in different settings. Importantly, these studies have revealed zone-specific DEC and agree that in the realm of enteric infections, watery diarrhea due

Table 1.1 Recent experimental vaccines developed against human ETEC infections

	Immunization protocol	Status	Comment	Selected references
dscCfaE, donor strand of fimbrial tip adhesin subunit of CFA/I	Intranasal and orogastric immunization of female BALB/c mice	Preclinical, animal model	Pilot immunization with donor strand complemented CfaE (dscCfaE) elicits robust hemagglutination inhibition and anti-adhesin antibody responses in both mice and nonhuman primate	Sincock et al. (2016)
	Intranasal immunization of the nonhuman primate *Aotus nancymaae* (4 males and 5 females)			
Single Multiple Pill® (SmPill®) vaccine, whole cell killed *E. coli* overexpressing CFA/I	Oral immunization of female Balb/c mice with nonliving oral JT-49 strain	Preclinical, animal model	Provoked CFA/I-specific IgA responses in the intestinal mucosa in addition to serum IgG	Davitt et al. (2016)
ETEC strain H10407A	Human mucosal immune responses were characterized and compared in subjects challenged with ETEC strain H10407 and in subjects rechallenged with the homologous organism	Early clinical development.	A dose of 10^7 CFU of ETEC strain H10407 administered to adult volunteers induced immune responses to LPS, LTB, and CFA/I in most of them. Some differences were appreciated in the immune response of challenged and rechallenged groups of volunteers, however, H10407 strain can be considered as an effective challenge model in ETEC vaccine studies	Chakraborty et al. (2016)
Detoxified outer membrane vesicles (OMVs) derived from *V. cholera*e and ETEC's CFA/I and FliC. OMVs can be easily isolated from different donor bacterial species and combined in various OMV mixtures	Intranasal immunization of female BALB/c mice was performed with *V. cholerae* as well as ETEC OMVs or with an OMV mixture	Preclinical animal model	Mice immunization with *V. cholerae* or ETEC OMVs induced a species-specific immune response, while the combination of both OMV species resulted in a high-titer, protective immune response against both pathogens	Leitner et al. (2015)
			Antibody responses against FliC and CFA/I heterologous expressed in OMV vehicles not improved the protection results obtained with OMV combination	

(continued)

Table 1.1 (continued)

	Immunization protocol	Status	Comment	Selected references
ETEC MltA-interacting protein MipA, the periplasmic chaperone	Intranasal immunization of female BALB/c mice	Preclinical, animal model	Immunization with ETEC conserved antigens showed improved cross-protective efficacy against heterologous ETEC producing different CFs. Mice that survived the challenge with H10407 had higher fecal IgA titers	Kumar et al. (2015)
Skp and a long-chain fatty acid outer membrane transporter, ETEC_2479				
Multi-epitope fusion antigen (MEFA) strategy to deliver epitopes of seven ETEC CFs: (CFA/I, CFA/II (CS1, CS2, CS3), CFA/IV (CS4, CS5, CS6) genetically fused to LT-STa toxoid	Intraperitoneal immunization female BALB/c mice	Preclinical, animal model	Induced antibodies that inhibited adherence in vitro of ETEC or *E. coli* strains expressing these seven CFs and neutralized activities of both toxins	Ruan et al. (2015)
Recombinant LT-A alone or in combination with LT-B-subunit	Intranasal immunization of female BALB/c mice	Preclinical animal model and clinical protocol with human intentionally or endemically infected with ETEC	Animals immunized with the recombinant proteins developed robust antibody responses that neutralizes the enterotoxic and cytotoxic effects of native LT by blocking binding and entry into cells (anti-LT-B) or the intracellular enzymatic activity of the toxin (anti-LT-A). Moreover, all individuals developed antibodies against both LT subunits	Norton et al. (2015)
	In addition, human individuals naturally infected with ETEC			
	Analysis of sera from subjects living in an ETEC-endemic area			

to ETEC continues to represent a serious health burden among young children in low-income communities. Considering the different tools available to each researcher to survey the presence of pathogens and their virulence factors, the studies provide important clues regarding the characteristics of the strains and the dynamics of pathogens and of virulence factors among endemic strains. The reduction of ETEC infections in some regions correlates with improvement of the region's economy and sanitary conditions. The availability of clean drinking water has made a marked impact in diminishing the rates of ETEC infections.

The epidemiological studies reviewed here vary substantially in design. For example, differences in settings and populations of study such as inclusion of matched case-controls, presence or absence of healthy controls, inclusion of hospitalized cases versus community or cohort cases, retrospective versus prospective studies, or whether there was a history or not of antibiotics therapy in the diarrheic children before or during the study. Not only these differences will impact the outcome, but they also make it difficult to establish fair comparisons between studies.

The implementation of standard protocols for the molecular diagnostic of *E. coli* pathotypes and for characterization of their virulence factors shall yield a closer approximation to the real prevalence of the different pathogens in the different settings and in the case of ETEC, of the presence of classical and nonclassical antigens. In all, these studies have unveiled to great extent the virulence characteristics of ETEC strains identifying toxin profiles, CFs, in some cases antibiotic resistance profiles, and age-associated attack rates of disease. The high detection rate of LT+ETEC among healthy controls remains an enigma. It is possible that genetic and nutritional host factors, the composition of the individual's microbiota, and fitness of the host immune system, play a modulatory role in determining the susceptibility of diarrheal cases to infections by more virulent ST+ and LT+/ST+ ETEC.

While monoclonal antibodies against known CFs and multiplex PCRs employing primers specific for known fimbrial subunits have been very helpful to identify known CFs in ETEC strains, these approaches miss identifying variants and new CFs. The search for fimbrial subunits genes using PCR alone has the disadvantage of potentially missing fimbrins, which under selective pressure of the immune system, may exhibit certain-to-high degree of variability. Employing primers specific for highly conserved chaperones or ushers of known fimbrial systems could expand our knowledge of new CFs in the so-called CF-negative ETEC. For the same reason, monoclonal antibodies could fail to detect detection epitopes in CF variants. The use of pools of polyclonal antibodies against individual CFs or sets of fimbrial families could help in this endeavor.

Most studies seeking CFs among ETEC, either by multiplex PCR or monoclonal antibodies, have identified the most common CFs: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17, CS21, and ECP. Considering the biochemical, structural, and antigenic similarities shared between the members of the CFA/I family as described in the text, the list can be reduced to six CFs namely, the CFA/I family, CS3, CS6, CS7, CS21, and ECP. This shorter list could perhaps help in the development of an ETEC CF-based vaccine with wider coverage. The studies also agree

that the frequency of CFs among ETEC falls within 40–50 %. This does not mean that the remaining strains do not produce fimbriae; we need to find them.

The plasticity of the *E. coli* genomes exemplified by the recent outbreaks of gut illness due to emergent hybrid strains (e.g., STEC/EAEC, STEC/ETEC) (Nyholm et al. 2015; Beutin and Martin 2012) should be a constant reminder of the need for efficient surveillance systems and the importance of the preparedness to detect hypervirulent variants in a timely manner.

Acknowledgments This research was supported by Fondecyt Grants 1110260.

References

Amezquita-Montes Z, Tamborski M, Kopsombut UG, Zhang C, Arzuza OS, Gomez-Duarte OG (2015) Genetic relatedness among *Escherichia coli* pathotypes isolated from food products for human consumption in Cartagena, Colombia. Foodborne Pathog Dis 12(5):454–461

Barry EM, Wang J, Wu T, Davis T, Levine MM (2006) Immunogenicity of multivalent *Shigella*-ETEC candidate vaccine strains in a guinea pig model. Vaccine 24(18):3727–3734

Bautista-De Leon H, Gomez-Aldapa CA, Rangel-Vargas E, Vazquez-Barrios E, Castro-Rosas J (2013) Frequency of indicator bacteria, *Salmonella* and diarrhoeagenic *Escherichia coli* pathotypes on ready-to-eat cooked vegetable salads from Mexican restaurants. Lett Appl Microbiol 56(6):414–420

Beutin L, Martin A (2012) Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot 75(2):408–418

Bhavnani D, Bayas Rde L, Lopez VK, Zhang L, Trueba G, Foxman B, Marrs C, Cevallos W, Eisenberg JN (2016) Distribution of enteroinvasive and enterotoxigenic *Escherichia coli* across space and time in Northwestern Ecuador. Am J Trop Med Hyg 94(2):276–284

Black RE (1990) Epidemiology of travelers' diarrhea and relative importance of various pathogens. Rev Infect Dis 12(suppl 1):S73–S79

Blackburn D, Husband A, Saldana Z, Nada RA, Klena J, Qadri F, Giron JA (2009) Distribution of the *Escherichia coli* common pilus among diverse strains of human enterotoxigenic E. coli. J Clin Microbiol 47(6):1781–1784

Castro-Rosas J, Cerna-Cortes JF, Mendez-Reyes E, Lopez-Hernandez D, Gomez-Aldapa CA, Estrada-Garcia T (2012) Presence of faecal coliforms, *Escherichia coli* and diarrheagenic E. coli pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. Int J Food Microbiol 156(2):176–180

Chakraborty S, Harro C, DeNearing B, Ram M, Feller A, Cage A, Bauers N, Bourgeois AL, Walker R, Sack DA (2016) Characterization of mucosal immune responses to enterotoxigenic *Escherichia coli* vaccine antigens in a human challenge model: response profiles after primary infection and homologous rechallenge with strain H10407. Clin Vaccine Immunol 23(1):55–64

Cote JP, Mourez M (2011) Structure-function analysis of the TibA self-associating autotransporter reveals a modular organization. Infect Immun 79(5):1826–1832

Cruz JR, Bartlett AV, Mendez H, Sibrian R (1992) Epidemiology of persistent diarrhea among Guatemalan rural children. Acta Paediatr 381:22–26

Cruz-Cordova A, Espinosa-Mazariego K, Ochoa SA, Saldana Z, Rodea GE, Cazares-Dominguez V, Rodriguez-Ramirez V, Eslava-Campos CA, Navarro-Ocana A, Arrellano-Galindo J, Hernandez-Castro R, Gomez-Duarte OG, Qadri F, Xicohtencatl-Cortes J (2014) CS21 positive multidrug-resistant ETEC clinical isolates from children with diarrhea are associated with self-aggregation, and adherence. Front Microbiol 5:709

Curtis V, Cairncross S, Yonli R (2000) Domestic hygiene and diarrhoea—pinpointing the problem. Trop Med Int Health 5(1):22–32

Davitt CJ, McNeela EA, Longet S, Tobias J, Aversa V, McEntee CP, Rosa M, Coulter IS, Holmgren J, Lavelle EC (2016) A novel adjuvanted capsule based strategy for oral vaccination against infectious diarrhoeal pathogens. J control Release 233:162–173

Del Canto F, Valenzuela P, Cantero L, Bronstein J, Blanco JE, Blanco J, Prado V, Levine M, Nataro J, Sommerfelt H, Vidal R (2011) Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J Clin Microbiol 49(9):3198–3203

Del Canto F, Botkin DJ, Valenzuela P, Popov V, Ruiz-Perez F, Nataro JP, Levine MM, Stine OC, Pop M, Torres AG, Vidal R (2012) Identification of coli surface antigen 23, a novel adhesin of enterotoxigenic *Escherichia coli*. Infect Immun 80(8):2791–2801

Diemert DJ (2006) Prevention and self-treatment of traveler's diarrhea. Clin Microbiol Rev 19(3):583–594

Dubreuil JD (2012) The whole Shebang: the gastrointestinal tract, *Escherichia coli* enterotoxins and secretion. Curr Issues Mol Biol 14(2):71–82

Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, Abonce M, Lopez-Hernandez D, Santos JI, Rosado JL, DuPont HL, Long KZ (2009) Association of diarrheagenic *Escherichia coli* pathotypes with infection and diarrhea among Mexican children and association of atypical Enteropathogenic *E. coli* with acute diarrhea. J Clin Microbiol 47(1):93–98

Evans DG, Silver RP, Evans DJ Jr, Chase DG, Gorbach SL (1975) Plasmid-controlled colonization factor associated with virulence in *Esherichia coli* enterotoxigenic for humans. Infect Immun 12(3):656–667

Fleckenstein JM, Sheikh A (2014) Designing vaccines to neutralize effective toxin delivery by enterotoxigenic *Escherichia coli*. Toxins 6(6):1799–1812

Fleckenstein JM, Kopecko DJ, Warren RL, Elsinghorst EA (1996) Molecular characterization of the tia invasion locus from enterotoxigenic *Escherichia coli*. Infect Immun 64(6):2256–2265

Fleckenstein JM, Lindler LE, Elsinghorst EA, Dale JB (2000) Identification of a gene within a pathogenicity island of enterotoxigenic *Escherichia coli* H10407 required for maximal secretion of the heat-labile enterotoxin. Infect Immun 68(5):2766–2774

Fleckenstein JM, Roy K, Fischer JF, Burkitt M (2006) Identification of a two-partner secretion locus of enterotoxigenic *Escherichia coli*. Infect Immun 74(4):2245–2258

Fleckenstein J, Sheikh A, Qadri F (2014) Novel antigens for enterotoxigenic *Escherichia coli* vaccines. Expert Rev Vaccines 13(5):631–639

Garcia C, Chincha O, Leon M, Iglesias D, Barletta F, Mercado E, Ochoa T (2010) High frequency of diarrheagenic *Escherichia coli* in human immunodeficiency virus (HIV) patients with and without diarrhea in Lima, Peru. Am J Trop Med Hyg 82(6):1118–1120

Garnett JA, Martinez-Santos VI, Saldana Z, Pape T, Hawthorne W, Chan J, Simpson PJ, Cota E, Puente JL, Giron JA, Matthews S (2012) Structural insights into the biogenesis and biofilm formation by the *Escherichia coli* common pilus. Proc Natl Acad Sci U S A 109(10): 3950–3955

Giron JA, Levine MM, Kaper JB (1994) Longus: a long pilus ultrastructure produced by human enterotoxigenic *Escherichia coli*. Mol Microbiol 12(1):71–82

Giron JA, Xu JG, Gonzalez CR, Hone D, Kaper JB, Levine MM (1995) Simultaneous expression of CFA/I and CS3 colonization factor antigens of enterotoxigenic *Escherichia coli* by delta aroC, delta aroD *Salmonella typhi* vaccine strain CVD 908. Vaccine 13(10):939–946

Gomez-Aldapa CA, Rangel-Vargas E, Bautista-De Leon H, Vazquez-Barrios ME, Gordillo-Martinez AJ, Castro-Rosas J (2013) Behavior of enteroaggregative *Escherichia coli*, non-O157-shiga toxin-producing *E. coli,* enteroinvasive *E. coli,* enteropathogenic E. coli and enterotoxigenic *E. coli* strains on mung bean seeds and and sprout. Int J Food Microbiol 166(3):364–368

Gomez-Aldapa CA, Rangel-Vargas E, Bautista-De Leon H, Castro-Rosas J (2014a) Presence of non-O157 Shiga toxin-producing *Escherichia coli*, enterotoxigenic *E. coli*, enteropathogenic

E. coli and *Salmonella* in fresh beetroot (Beta vulgaris L.) juice from public markets in Mexico. J Sci Food Agric 94(13):2705–2711

Gomez-Aldapa CA, Rangel-Vargas E, Gordillo-Martinez AJ, Castro-Rosas J (2014b) Behavior of shiga toxin-producing *Escherichia coli*, enteroinvasive *E. coli*, enteropathogenic E. coli and enterotoxigenic E. coli strains on whole and sliced jalapeno and serrano peppers. Food Microbiol 40:75–80

Gomez-Duarte OG, Arzuza O, Urbina D, Bai J, Guerra J, Montes O, Puello M, Mendoza K, Castro GY (2010) Detection of *Escherichia coli* enteropathogens by multiplex polymerase chain reaction from children's diarrheal stools in two Caribbean-Colombian cities. Foodborne Pathog Dis 7(2):199–206

Gomez-Duarte OG, Romero-Herazo YC, Paez-Canro CZ, Eslava-Schmalbach JH, Arzuza O (2013) Enterotoxigenic *Escherichia coli* associated with childhood diarrhoea in Colombia, South America. J Infect Dev Ctries 7(5):372–381

Gonzaga VE, Ramos M, Maves RC, Freeman R, Montgomery JM (2011) Concurrent outbreak of norovirus genotype I and enterotoxigenic *Escherichia coli* on a U.S. Navy ship following a visit to Lima, Peru. PLoS One 6(6), e20822

Gonzales L, Joffre E, Rivera R, Sjoling A, Svennerholm AM, Iniguez V (2013a) Prevalence, seasonality and severity of disease caused by pathogenic *Escherichia coli* in children with diarrhoea in Bolivia. J Med Microbiol 62(pt 11):1697–1706

Gonzales L, Sanchez S, Zambrana S, Iniguez V, Wiklund G, Svennerholm AM, Sjoling A (2013b) Molecular characterization of enterotoxigenic *Escherichia coli* isolates recovered from children with diarrhea during a 4-year period (2007 to 2010) in Bolivia. J Clin Microbiol 51(4):1219–1225

Guerra JA, Romero-Herazo YC, Arzuza O, Gomez-Duarte OG (2014) Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* clinical isolates from northern Colombia, South America. BioMed Res Int 2014:236260

Gutierrez D, Pardo M, Montero D, Onate A, Farfan MJ, Ruiz-Perez F, Del Canto F, Vidal R (2015) TleA, a Tsh-like autotransporter identified in a human enterotoxigenic *Escherichia coli* strain. Infect Immun 83(5):1893–1903

Gutierrez-Cazarez Z, Qadri F, Albert MJ, Giron JA (2000) Identification of enterotoxigenic *Escherichia coli* harboring longus type IV pilus gene by DNA amplification. J Clin Microbiol 38(5):1767–1771

Honda T, Arita M, Miwatani T (1984) Characterization of new hydrophobic pili of human enterotoxigenic *Escherichia coli*: a possible new colonization factor. Infect Immun 43(3):959–965

Jansson L, Tobias J, Jarefjall C, Lebens M, Svennerholm AM, Teneberg S (2009) Sulfatide recognition by colonization factor antigen CS6 from enterotoxigenic *Escherichia coli*. PLoS One 4(2), e4487

Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2(2):123–140

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382(9888):209–222

Kumar P, Luo Q, Vickers TJ, Sheikh A, Lewis WG, Fleckenstein JM (2014) EatA, an immunogenic protective antigen of enterotoxigenic *Escherichia coli*, degrades intestinal mucin. Infect Immun 82(2):500–508

Kumar A, Hays M, Lim F, Foster LJ, Zhou M, Zhu G, Miesner T, Hardwidge PR (2015) Protective enterotoxigenic *Escherichia coli* antigens in a murine intranasal challenge model. PLoS Negl Trop Dis 9(8), e0003924

Leitner DR, Lichtenegger S, Temel P, Zingl FG, Ratzberger D, Roier S, Schild-Prufert K, Feichter S, Reidl J, Schild S (2015) A combined vaccine approach against Vibrio cholerae and ETEC based on outer membrane vesicles. Front Microbiol 6:823

Levine MM, Ristaino P, Marley G, Smyth C, Knutton S, Boedeker E, Black R, Young C, Clements ML, Cheney C et al (1984) Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. Infect Immun 44(2):409–420

Levine MM, Ferreccio C, Prado V, Cayazzo M, Abrego P, Martinez J, Maggi L, Baldini MM, Martin W, Maneval D et al (1993) Epidemiologic studies of *Escherichia coli* diarrheal infections in a low socioeconomic level peri-urban community in Santiago, Chile. Am J Epidemiol 138(10):849–869

Lindenthal C, Elsinghorst EA (1999) Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. Infect Immun 67(8):4084–4091

Lonardi E, Moonens K, Buts L, de Boer AR, Olsson JD, Weiss MS, Fabre E, Guerardel Y, Deelder AM, Oscarson S, Wuhrer M, Bouckaert J (2013) Structural sampling of glycan interaction profiles reveals mucosal receptors for fimbrial adhesins of enterotoxigenic *Escherichia coli*. Biology 2(3):894–917

Lozer DM, Souza TB, Monfardini MV, Vicentini F, Kitagawa SS, Scaletsky IC, Spano LC (2013) Genotypic and phenotypic analysis of diarrheagenic *Escherichia coli* strains isolated from Brazilian children living in low socioeconomic level communities. BMC Infect Dis 13:418

Ludwig A, von Rhein C, Bauer S, Huttinger C, Goebel W (2004) Molecular analysis of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. J Bacteriol 186(16):5311–5320

Luo Q, Kumar P, Vickers TJ, Sheikh A, Lewis WG, Rasko DA, Sistrunk J, Fleckenstein JM (2014) Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect Immun 82(2):509–521

Madhavan TP, Sakellaris H (2015) Colonization factors of enterotoxigenic *Escherichia coli*. Adv Appl Microbiol 90:155–197

Madhavan TP, Riches JD, Scanlon MJ, Ulett GC, Sakellaris H (2016) Binding of CFA/I Pili of enterotoxigenic *Escherichia coli* to Asialo-GM1 is mediated by the Minor Pilin, CfaE. Infect Immun 84:1642–1649

McLean M, Brennan R, Hughes JM, Korzeniowski OM, de Souza MA, Araujo JG, Benevides TM, Guerrant RL (1981) Etiology of childhood diarrhea and oral rehydration therapy in northeastern Brazil. Bull Pan Am Health Organ 15(4):318–325

Medina AM, Rivera FP, Pons MJ, Riveros M, Gomes C, Bernal M, Meza R, Maves RC, Huicho L, Chea-Woo E, Lanata CF, Gil AI, Ochoa TJ, Ruiz J (2015) Comparative analysis of antimicrobial resistance in enterotoxigenic *Escherichia coli* isolates from two paediatric cohort studies in Lima, Peru. Trans R Soc Trop Med Hyg 109(8):493–502

Moreno AC, Filho AF, Gomes Tdo A, Ramos ST, Montemor LP, Tavares VC, Filho Ldos S, Irino K, Martinez MB (2010) Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. Diagn Microbiol Infect Dis 66(1):50–57

Mudrak B, Kuehn MJ (2010) Heat-labile enterotoxin: beyond G(m1) binding. Toxins 2(6):1445–1470

Nair P, Okhuysen PC, Jiang ZD, Carlin LG, Belkind-Gerson J, Flores J, Paredes M, DuPont HL (2014) Persistent abdominal symptoms in US adults after short-term stay in Mexico. J Travel Med 21(3):153–158

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11(1):142–201

Norton EB, Branco LM, Clements JD (2015) Evaluating the A-subunit of the heat-labile toxin (LT) as an immunogen and a protective antigen against enterotoxigenic *Escherichia coli* (ETEC). PLoS One 10(8), e0136302

Novoa-Farias O, Frati-Munari AC, Peredo MA, Flores-Juarez S, Novoa-Garcia O, Galicia-Tapia J, Romero-Carpio CE (2016) Susceptibility of bacteria isolated from acute gastrointestinal infections to rifaximin and other antimicrobial agents in Mexico. Rev Gastroenterol Mex 81(1):3–10

Nyholm O, Halkilahti J, Wiklund G, Okeke U, Paulin L, Auvinen P, Haukka K, Siitonen A (2015) Comparative genomics and characterization of hybrid shigatoxigenic and enterotoxigenic *Escherichia coli* (STEC/ETEC) strains. PLoS One 10(8), e0135936

O'Ryan M, Vidal R, del Canto F, Carlos Salazar J, Montero D (2015) Vaccines for viral and bacterial pathogens causing acute gastroenteritis: part II: vaccines for *Shigella*, *Salmonella*, enterotoxigenic *E. coli* (ETEC) enterohemorragic *E. coli* (EHEC) and *Campylobacter jejuni*. Hum Vaccines Immunother 11(3):601–619

Ouyang-Latimer J, Ajami NJ, Jiang ZD, Okhuysen PC, Paredes M, Flores J, Dupont HL (2010) Biochemical and genetic diversity of enterotoxigenic *Escherichia coli* associated with diarrhea in United States students in Cuernavaca and Guadalajara, Mexico, 2004-2007. J Infect Dis 201(12):1831–1838

Ouyang-Latimer J, Jafri S, VanTassel A, Jiang ZD, Gurleen K, Rodriguez S, Nandy RK, Ramamurthy T, Chatterjee S, McKenzie R, Steffen R, DuPont HL (2011) In vitro antimicrobial susceptibility of bacterial enteropathogens isolated from international travelers to Mexico, Guatemala, and India from 2006 to 2008. Antimicrob Agents Chemother 55(2):874–878

Paredes-Paredes M, Okhuysen PC, Flores J, Mohamed JA, Padda RS, Gonzalez-Estrada A, Haley CA, Carlin LG, Nair P, DuPont HL (2011) Seasonality of diarrheagenic *Escherichia coli* pathotypes in the US students acquiring diarrhea in Mexico. J Travel Med 18(2):121–125

Patzi-Vargas S, Zaidi MB, Perez-Martinez I, Leon-Cen M, Michel-Ayala A, Chaussabel D, Estrada-Garcia T (2015) Diarrheagenic *Escherichia coli* carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis 9(3), e0003510

Pouttu R, Westerlund-Wikstrom B, Lang H, Alsti K, Virkola R, Saarela U, Siitonen A, Kalkkinen N, Korhonen TK (2001) matB, a common fimbrillin gene of *Escherichia coli*, expressed in a genetically conserved, virulent clonal group. J Bacteriol 183(16):4727–4736

Qadri F, Giron JA, Helander A, Begum YA, Asaduzzaman M, Xicohtencatl-Cortes J, Negrete E, Albert MJ (2000) Human antibody response to longus type IV pilus and study of its prevalence among enterotoxigenic *Escherichia coli* in Bangladesh by using monoclonal antibodies. J Infect Dis 181(6):2071–2074

Qadri F, Svennerholm AM, Faruque AS, Sack RB (2005) Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18(3):465–483

Rangel-Vargas E, Gomez-Aldapa CA, Torres-Vitela Mdel R, Villarruel-Lopez A, Gordillo-Martinez AJ, Castro-Rosas J (2015) Presence and correlation of some enteric indicator bacteria, diarrheagenic *Escherichia coli* pathotypes, and *Salmonella* serotypes in alfalfa sprouts from local retail markets in Pachuca, Mexico. J Food Prot 78(3):609–614

Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, Paxinos EE, Sebra R, Chin CS, Iliopoulos D, Klammer A, Peluso P, Lee L, Kislyuk AO, Bullard J, Kasarskis A, Wang S, Eid J, Rank D, Redman JC, Steyert SR, Frimodt-Moller J, Struve C, Petersen AM, Krogfelt KA, Nataro JP, Schadt EE, Waldor MK (2011) Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. N Engl J Med 365(8):709–717

Rausch D, Ruan X, Nandre R, Duan Q, Hashish E, Casey TA, Zhang W (2016) Antibodies derived from a toxoid MEFA (multiepitope fusion antigen) show neutralizing activities against heat-labile toxin (LT), heat-stable toxins (STa, STb), and Shiga toxin 2e (Stx2e) of porcine enterotoxigenic *Escherichia coli* (ETEC). Vet Microbiol 2016 Feb 6. pii: S0378-1135(16)30027-X

Rendon MA, Saldana Z, Erdem AL, Monteiro-Neto V, Vazquez A, Kaper JB, Puente JL, Giron JA (2007) Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. Proc Natl Acad Sci U S A 104(25):10637–10642

Reyes D, Vilchez S, Paniagua M, Colque-Navarro P, Weintraub A, Mollby R, Kuhn I (2010) Diarrheagenic *Escherichia coli* markers and phenotypes among fecal *E. coli* isolates collected from Nicaraguan infants. J Clin Microbiol 48(9):3395–3396

Rivera FP, Ochoa TJ, Maves RC, Bernal M, Medina AM, Meza R, Barletta F, Mercado E, Ecker L, Gil AI, Hall ER, Huicho L, Lanata CF (2010) Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. J Clin Microbiol 48(9):3198–3203

Rivera FP, Medina AM, Aldasoro E, Sangil A, Gascon J, Ochoa TJ, Vila J, Ruiz J (2013) Genotypic characterization of enterotoxigenic *Escherichia coli* strains causing traveler's diarrhea. J Clin Microbiol 51(2):633–635

Rodas C, Klena JD, Nicklasson M, Iniguez V, Sjoling A (2011a) Clonal relatedness of enterotoxigenic *Escherichia coli* (ETEC) strains expressing LT and CS17 isolated from children with diarrhoea in La Paz, Bolivia. PLoS One 6(11), e18313

Rodas C, Mamani R, Blanco J, Blanco JE, Wiklund G, Svennerholm AM, Sjoling A, Iniguez V (2011b) Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. Braz J Infect Dis 15(2):132–137

Roy K, Hamilton D, Allen KP, Randolph MP, Fleckenstein JM (2008) The EtpA exoprotein of enterotoxigenic *Escherichia coli* promotes intestinal colonization and is a protective antigen in an experimental model of murine infection. Infect Immun 76(5):2106–2112

Roy K, Hilliard GM, Hamilton DJ, Luo J, Ostmann MM, Fleckenstein JM (2009) Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. Nature 457(7229):594–598

Roy K, Kansal R, Bartels SR, Hamilton DJ, Shaaban S, Fleckenstein JM (2011) Adhesin degradation accelerates delivery of heat-labile toxin by enterotoxigenic *Escherichia coli*. J Biol Chem 286(34):29771–29779

Ruan X, Sack DA, Zhang W (2015) Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) retain broad anti-CFA and antitoxin antigenicity. PLoS One 10(3), e0121623

Rugeles LC, Bai J, Martinez AJ, Vanegas MC, Gomez-Duarte OG (2010) Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. Int J Food Microbiol 138(3):282–286

Sanchez J, Holmgren J (2005) Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea. Curr Opin Immunol 17(4):388–398

Sheikh A, Luo Q, Roy K, Shabaan S, Kumar P, Qadri F, Fleckenstein JM (2014) Contribution of the highly conserved EaeH surface protein to enterotoxigenic *Escherichia coli* pathogenesis. Infect Immun 82(9):3657–3666

Sherlock O, Vejborg RM, Klemm P (2005) The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. Infect Immun 73(4):1954–1963

Sincock SA, Hall ER, Woods CM, O'Dowd A, Poole ST, McVeigh AL, Nunez G, Espinoza N, Miller M, Savarino SJ (2016) Immunogenicity of a prototype enterotoxigenic *Escherichia coli* adhesin vaccine in mice and nonhuman primates. Vaccine 34(2):284–291

Smyth CJ (1982) Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H-isolated from travellers' and infantile diarrhoea. J Gen Microbiol 128(9):2081–2096

Spangler BD (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 56(4):622–647

Steffen R, Castelli F, Dieter Nothdurft H, Rombo L, Jane Zuckerman N (2005) Vaccination against enterotoxigenic *Escherichia coli*, a cause of travelers' diarrhea. J Travel Med 12(2):102–107

Thomas LV, Cravioto A, Scotland SM, Rowe B (1982) New fimbrial antigenic type (E8775) that may represent a colonization factor in enterotoxigenic *Escherichia coli* in humans. Infect Immun 35(3):1119–1124

Thomas LV, McConnell MM, Rowe B, Field AM (1985) The possession of three novel coli surface antigens by enterotoxigenic *Escherichia coli* strains positive for the putative colonization factor PCF8775. J Gen Microbiol 131(9):2319–2326

Toma C, Higa N, Iyoda S, Rivas M, Iwanaga M (2006) The long polar fimbriae genes identified in Shiga toxin-producing *Escherichia coli* are present in other diarrheagenic *E. coli* and in the standard *E. coli* collection of reference (ECOR) strains. Res Microbiol 157(2):153–161

Torres AG, Giron JA, Perna NT, Burland V, Blattner FR, Avelino-Flores F, Kaper JB (2002) Identification and characterization of lpfABCC'DE, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 70(10):5416–5427

Torres OR, Gonzalez W, Lemus O, Pratdesaba RA, Matute JA, Wiklund G, Sack DA, Bourgeois AL, Svennerholm AM (2015) Toxins and virulence factors of enterotoxigenic *Escherichia coli* associated with strains isolated from indigenous children and international visitors to a rural community in Guatemala. Epidemiol Infect 143(8):1662–1671

Torres-Vitela MD, Gomez Aldapa CA, Cerna-Cortes JF, Villarruel-Lopez A, Rangel-Vargas E, Castro-Rosas J (2013) Presence of indicator bacteria, diarrhoeagenic *Escherichia coli* pathotypes and *Salmonella* in fresh carrot juice from Mexican restaurants. Lett Appl Microbiol 56(3):180–185

Turner SM, Chaudhuri RR, Jiang ZD, DuPont H, Gyles C, Penn CW, Pallen MJ, Henderson IR (2006) Phylogenetic comparisons reveal multiple acquisitions of the toxin genes by enterotoxigenic *Escherichia coli* strains of different evolutionary lineages. J Clin Microbiol 44(12):4528–4536

Vidal R, Vidal M, Lagos R, Levine M, Prado V (2004) Multiplex PCR for diagnosis of enteric infections associated with diarrheagenic *Escherichia coli*. J Clin Microbiol 42(4):1787–1789

Vidal M, Kruger E, Duran C, Lagos R, Levine M, Prado V, Toro C, Vidal R (2005) Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. J Clin Microbiol 43(10):5362–5365

Zhang W, Sack DA (2015) Current progress in developing subunit vaccines against enterotoxigenic *Escherichia coli*-associated diarrhea. Clin Vaccine Immunol 22(9):983–991

Chapter 2
Enteroaggregative *Escherichia coli* (EAEC)

Waldir P. Elias and Fernando Navarro-Garcia

Summary Enteroaggregative *Escherichia coli* (EAEC) is defined by the production of the characteristic aggregative adherence pattern on cultured epithelial cells. This pathotype is a food-borne emerging enteropathogen, responsible for cases of acute and persistent diarrhea in children and immunocompromised patients in developing countries, as well as in travelers returning from endemic areas. Growth and cognitive impairment are linked to EAEC infections in children living in developing countries. The pathogenesis of EAEC is characterized by abundant adherence to the intestinal mucosa, elaboration of enterotoxins/cytotoxins, and induction of mucosal inflammation. Several putative virulence factors associated with these three steps have been identified and characterized, but none of them is present in all strains. The virulence gene(s) that define virulent strains within this complex heterogeneity is yet to be determined. An increasing attention to this pathotype emerged since the massive outbreak caused by a hybrid hypervirulent Shiga toxin-expressing EAEC strain with severe clinical consequences.

W.P. Elias
Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil
e-mail: waldir.elias@butantan.gov.br

F. Navarro-Garcia (✉)
Department of Cell Biology, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México, Distrito Federal, Mexico
e-mail: fnavarro@cell.cinvestav.mx

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_2

1 General Concepts

1.1 Defining EAEC

The term aggregative adherence was coined by Nataro and colleagues when examining the adherence properties of *E. coli* strains isolated in an epidemiological study of childhood diarrhea in the city of Santiago, Chile (Nataro et al. 1987). The isolates were tested for interaction with HEp-2 cells, and three adherence patterns were described. In addition to the previously described localized adherence (LA) pattern, the term "diffuse adherence" was distinguished into the truly diffuse adhesion (DA) and the aggregative adherence (AA). Standard AA is defined as bacteria adhering to each other, on the surface of epithelial cells as well as on the surface of the coverslip in the absence of cells (Fig. 2.1). Such configuration resembles stacked bricks forming heterogeneous aggregate or distributed in chains (Hebbelstrup Jensen et al. 2014).

Strains expressing the AA pattern were then called "enteroadherent-aggregative *E. coli,*" but this term was replaced by the current name of enteroaggregative *E. coli*, or EAEC (Estrada-Garcia and Navarro-Garcia 2012). The AA phenotype is *sine qua non* to classify an *E. coli* strain as belonging to the category of EAEC. Nowadays, the most appropriate and updated definition of EAEC encompasses strains that produce

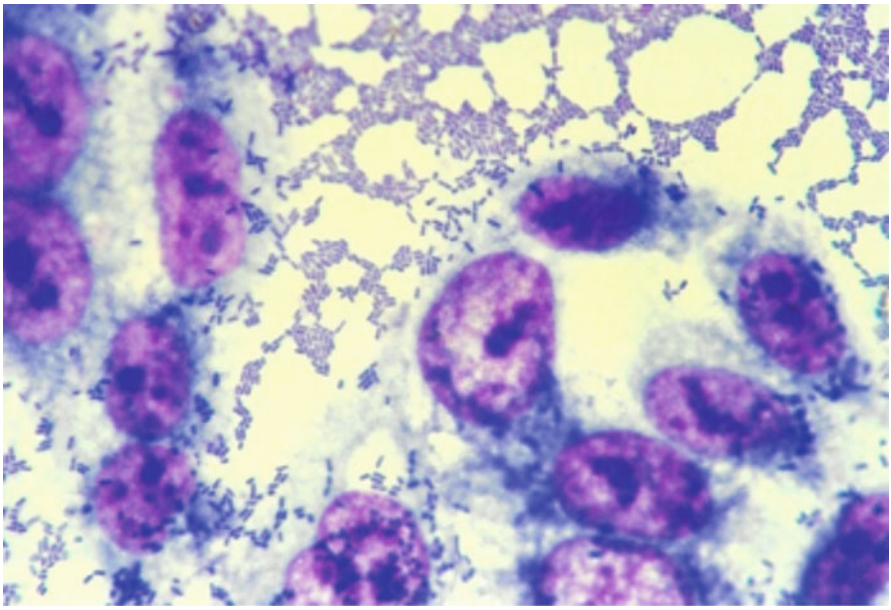

Fig. 2.1 Characteristic aggregative adherence (AA) pattern of EAEC on HEp-2 cells. EAEC prototypical strain 042 was incubated with epithelial cells for 3 h and the preparation was stained with May-Grünwald and Giemsa

the AA pattern on HeLa or HEp-2 cells (3 or 6 h adhesion assay) and are devoid of virulence markers that define other types of diarrheagenic *E. coli*. An exception is for strains presenting the AA pattern and other EAEC-specific genetic markers in combination with the production of Shiga toxin (Stx), which defines the hybrid EAEC and Shiga toxin-producing *E. coli* (STEC), discussed below.

Currently, EAEC is considered an emerging enteropathogen, responsible for cases of acute and persistent diarrhea in children and adults worldwide, and developmental consequences in children living in developing countries (Hebbelstrup Jensen et al. 2014). An increasing attention to this pathotype has arisen from the massive outbreak caused by a hybrid hypervirulent EAEC strain (Stx2-expressing EAEC) with severe sequelae such as the development of hemolytic uremic syndrome (Navarro-Garcia 2014).

1.2 General Epidemiology

Since its description in 1987, when EAEC was significantly associated with acute diarrhea in children (Nataro et al. 1987), numerous epidemiological studies of the etiology of diarrhea searched for EAEC in an attempt to clarify its role as diarrhea agent. In the early years, the association between EAEC and persistent diarrhea ($\geq$14 days of duration) in children was well supported (Cravioto et al. 1991). However, the association with acute diarrhea in childhood was controversial.

In the following years, a large number of studies have reported the detection of EAEC in cases of acute diarrhea in developing and developed countries, persistent diarrhea in developing countries, and significant outbreaks worldwide. In fact, Huang and colleagues demonstrated by a meta-analysis study of the literature between 1987 and 2006 that EAEC was statistically associated with acute and persistent diarrhea in developed and developing countries, to diarrhea in HIV-infected patients in developing countries, and adults traveler's diarrhea (Huang et al. 2006). Another recent meta-analysis study of published articles between 1989 and 2011 showed association of EAEC with acute diarrhea in children of South Asian populations (Pabalan et al. 2013). Thus, EAEC has been systematically identified as an emerging enteropathogen, globally distributed (Estrada-Garcia and Navarro-Garcia 2012).

A high rate of asymptomatic young children carrying EAEC is still a trend reported in several studies in the last years (Hebbelstrup Jensen et al. 2014). Moreover, such persistent colonization has a link with growth impairment in children from low socioeconomic status.

The linkage between EAEC and diarrhea in individuals living in developed countries became clearer in the last years. In the USA and Europe, EAEC has been frequently isolated from cases of diarrhea from children and adults. In prospective studies, EAEC was the major cause of diarrhea in children in the USA (Nataro et al. 2006).

Traveler's diarrhea (TD) is the most frequent disease that affects individuals living in developed countries when visiting middle and low incoming endemic areas. EAEC has been systematically found among the most prevalent bacterial agents of

traveler's diarrhea since the definition of this category. The prevalence of EAEC in TD varies from 19 to 33 %, depending on the geographic region visited (Mohamed et al. 2011).

Studies linked EAEC with diarrhea in HIV-infected adults and children, a group usually susceptible to significant cases of protracted diarrhea. EAEC was isolated as the only enteropathogen in symptomatic patients presenting diarrhea for 30 days (Polotsky et al. 1997). In addition, isolation of EAEC was similar in a case/control study of HIV patients (Medina et al. 2010).

Several outbreaks of gastroenteritis caused by EAEC have been reported in low- and high-income countries. Some of them involving very impressive numbers of infected children or adults and associated with the consumption of contaminated food. Outbreaks in the UK (Dallman et al. 2014), Japan (Itoh et al. 1997), and Italy (Scavia et al. 2008) show the relevance of EAEC in developed countries. In one of the Japanese outbreaks, 2697 schoolchildren were affected after consumption of school lunches (Itoh et al. 1997). In Italy, the outbreaks were transmitted by unpasteurized cheese. EAEC was also responsible for outbreaks in developing countries (Cobeljic et al. 1996).

1.3 Clinical Features

The most common symptoms reported in EAEC infection are watery diarrhea, often mucoid, with or without blood and abdominal pain, nausea, vomiting, and low fever. These signs and symptoms are often self-limited but some selected patients may develop persistent diarrhea (≥14 days). The diversity of clinical symptoms in this pathotype infection may be due to heterogeneity between EAEC isolates, infectious dose, genetic susceptibility factors in the host, as well as the immune response (Harrington et al. 2006).

In order to better characterize the virulent properties of EAEC, studies with human volunteers receiving oral inoculum of different EAEC strains were performed (Mathewson et al. 1986; Nataro et al. 1992, 1995).

Nataro and colleagues evaluated four different EAEC isolated from different geographic regions and different serotypes in volunteers (Nataro et al. 1995). In this study, the volunteers ingested dose of 10^{10} CFU and just EAEC O42 (serotype O44:H18) caused diarrhea in three out of five volunteers. EAEC 042 was isolated from a case of childhood diarrhea in Peru. The clinical data obtained from the volunteers who developed diarrhea suggested that EAEC 042 caused secretory diarrhea, with short incubation period, absence of fever, and leukocytes or blood in the stool. Furthermore, the mucus found in the feces of two patients suggested larger intestinal secretion induced by colonization by EAEC 042. These studies, as well as others that demonstrate the virulence of EAEC for humans, have indicated that EAEC is a heterogeneous category of diarrheagenic *E. coli*, including virulent strains or not, what seems to depend on factors not yet fully understood.

1.4 Histopathology

Studies in vitro, in vivo, and ex vivo, evaluating EAEC interactions with intestinal cells from animals or humans, have tried to elucidate the pathogenesis of this pathotype since its description in 1987. The first study about EAEC pathogenicity in animal models employed ligated ileal loop of rabbit and rat intestines (Vial et al. 1988), showing that EAEC 042 and 17-2 strongly adhered to the mucosa and causing shortening of microvilli, hemorrhagic necrosis with edema, and mononuclear infiltrates in the submucosa. Analysis by transmission electron microscopy revealed no bacterial invasion and the microvilli architecture was preserved.

Other studies employing in vitro organ culture (IVOC) models have elucidated the intestinal alterations induced by EAEC in fragments of human biopsies of duodenum, ileum, and colon (Hicks et al. 1996; Nataro et al. 1996). Cytotoxic effects in the colon were observed, such as microvilli vesiculation, enlarged crypt openings, and increased epithelial cell extrusion (Hicks et al. 1996). Also using IVOC, Nataro and colleagues demonstrated strong adhesion of EAEC 042 to jejunal mucosa, ileum, and more intensely to the colon, while 042 cured of the pAA2 plasmid lost this ability (Nataro et al. 1996). All together, these data strongly indicated that EAEC virulence was probable due to intestinal colonization, mainly to the colonic mucosa, in the characteristic aggregative manner, with bacteria forming strong biofilm in an increased mucus layer, followed by cytotoxic and pro-inflammatory effects.

1.5 Strain Heterogeneity

In the decades that followed its original description, EAEC strains have been characterized in numerous studies around the world, highlighting the particular heterogeneity of this category in terms of serotypes, genetic determinants linked to virulence and phylogenetic groups (Boisen et al. 2012; Chattaway et al. 2014b; Czeczulin et al. 1999; Jenkins et al. 2006). This heterogeneity together with the fact that not all EAEC strains were able to cause diarrhea in experimental infection of humans raises the idea that only a subset of EAEC strains, carrying a specific set of virulence factors, has the capacity to cause diarrhea. This set of factors has not been determined.

After demonstrating its pathogenicity in human volunteers, EAEC 042 became genetically and phenotypically widely studied and considered the prototype strain for EAEC. Major advances in understanding the pathogenesis of EAEC resulted from data obtained with this strain whose genome has been sequenced (Chaudhuri et al. 2010). Other EAEC strains have been also used as prototype in studies describing virulence factors and pathogenic mechanisms not present in EAEC 042. Despite the variety of identified virulence factors, such as enterotoxins, cytotoxins, secreted proteins, outer membrane proteins, and fimbriae (Table 2.1), the pathogenesis of the diarrhea caused by EAEC remains unclear.

Table 2.1 EAEC prototype strains and their main virulence factors

Prototype strain (serotype)	Virulence factor	Genes	References
042 (O44:H18)	AggR—aggregative master regulator	*aggR*	Morin et al. (2013)
	Shf—*Shigella flexneri* homologue involved in biofilm formation	*shf*	Czeczulin et al. (1999)
	VirK—*Shigella flexneri* homologue Pet chaperone	*virK*	Tapia-Pastrana et al. (2012)
	CapU—hexosyltransferase homologue	*capU*	Czeczulin et al. (1999)
	ABC transporter system—dispersin transporter system	aatA	Nishi et al. (2003)
	AAF/II—aggregative adherence fimbria II	*aafABCD*	Elias et al. (1999)
	Pet—plasmid-encoded toxin	*pet*	Eslava et al. (1998)
	Pic—protein involved in colonization	*pic*	Henderson et al. (1999a)
	EAST-1—aggregative heat-stable toxin 1	*astA*	Savarino et al. (1991)
	Dispersin—anti-aggregation protein	*aap*	Sheikh et al. (2002)
	Type VI secretion system	*aaiA-Y*	Dudley et al. (2006b)
	ETT2 *Escherichia coli* type three secretion system 2	*eprHIJK, etrA, eivH, epaOPQRS, eivFGEACIJ*	Sheikh et al. (2006)
	Air—enteroaggregative immunoglobulin repeat protein	*air*	Sheikh et al. (2006)
	EilA—*Salmonella HilA regulator* homologue	*eilA*	Sheikh et al. (2006)
	Orf61—hypothetical plasmid-encoded hemolysin	*orf61*	Chaudhuri et al. (2010)
	Ag43—phase-variable antigen 43	*agn43*	Chaudhuri et al. (2010)
	Hra1—heat-resistant agglutinin 1	*hra1*	Bhargava et al. (2009)
17-2 (O3:H2)	AAF/I—aggregative adherence fimbria I	*aggABCD*	Nataro et al. (1992)
55989 (O104:H4)	AAF/III—aggregative adherence fimbria III	*agg3ABCD*	Bernier et al. (2002)
C1010-00 (OR:H1)	AAF/IV—aggregative adherence fimbria IV	*agg4ABCD*	Boisen et al. (2008)

(continued)

Table 2.1 (continued)

Prototype strain (serotype)	Virulence factor	Genes	References
C338-14 (O55:H19)	AAF/V—aggregative adherence fimbria V	*Agg5ABCD*	Jonsson et al. (2015)
C1096 (O4:HNT)	Pil—type IV pilus	*pilLMNOPQRSTUV*	Dudley et al. (2006a)
JM221 (O92:H33)	AAF/I—aggregative adherence fimbria I	*aggABCD*	Mathewson et al. (1986)
60A (ND)	Hra2—heat-resistant agglutinin 2	*hra2*	Mancini et al. (2011)

OR O rough, *NT* non-typable, *ND* not determined

Due to the association of AA phenotype with high-molecular weight plasmids carrying large number of plasmid-encoded virulence factors in EAEC, these plasmids are called aggregative virulence plasmids or pAA (Harrington et al. 2006).

From the pAA1 plasmid present in the prototype EAEC 17-2 (serotype O3:H2) Baudry et al. (1990) isolated the CVD432 probe fragment, widely used for the molecular diagnosis of EAEC. Plasmid pAA2, present in the EAEC 042, also has approximately 100 kb of genetic information and encodes many well-characterized virulence factors (Czeczulin et al. 1999).

AggR is a transcriptional activator and regulates the expression of various virulence factors present in the chromosome and pAA2 plasmid of EAEC 042, defining the AggR regulon (Harrington et al. 2006). At least 44 genes are regulated by *aggR*, including the genes for AAF/II biogenesis, dispersin and its secretion system, Shf, CapU, the *aai* type VI secretion system, and *aagR* itself (Morin et al. 2013; Dudley et al. 2006b). Not all EAEC strains harbor *aggR* and, consequently, the pAA plasmid. Thus, the classification of EAEC was proposed into two subgroups, e.g., typical and atypical, taking into account the presence or absence of *aggR*, respectively (Harrington et al. 2006). This classification defines two groups of strains, one of them consisting of typical strains with higher pathogenic potential due to the presence of the AggR regulon and the pAA virulence plasmid (Estrada-Garcia and Navarro-Garcia 2012). However, atypical EAEC strains are commonly isolated from cases of diarrhea, as the sole pathogen (Huang et al. 2007; Jiang et al. 2002). In one epidemiological study on the etiology of acute diarrhea in children from Espírito Santo (Brazil), atypical strains were more frequent than typical strains (Isabel Scaletsky, unpublished data). In addition, at least two outbreaks of diarrhea were caused by atypical EAEC (Cobeljic et al. 1996), one of them affected more than two thousand children (Itoh et al. 1997).

1.6 Pathogenesis in Three Steps

EAEC ability to mediate diarrhea was clearly established through the volunteer study with EAEC strain 042 (Nataro et al. 1995). However, this study and others have left clear that the pathogenesis of EAEC is complex, and EAEC strains are very heterogeneous.

Data accumulated from several studies have suggested three major features of EAEC pathogenesis: (1) abundant adherence to the intestinal mucosa, (2) elaboration of enterotoxins and cytotoxins, and (3) induction of mucosal inflammation (Fig. 2.2). These stages of EAEC pathogenicity have been obtained from studies in vitro in cell cultures, animal models, and patients infected with EAEC (Hicks et al. 1996; Navarro-Garcia and Elias 2011). Heterogeneity is also found in EAEC colonization, because once ingested, the location of infection in the gastrointestinal tract has not been well defined. Studies done on endoscopic intestinal specimens demonstrate that EAEC can bind to jejunal, ileal, and colonic epithelium (Nataro et al. 1996). These findings were recently validated in fragments from terminal ileum and colon that were excised from pediatric patients undergoing intestinal surgeries and from adult patients that underwent colonoscopy treatment; such intestinal segments were used to define interaction with three EAEC strains. These bacteria colonized ileal and colonic mucosa in the typical stacked-brick configuration. In both regions, the strains were seen over a great amount of mucus and sometimes over the intact epithelium. It was possible to see adhered bacteria to the intestinal surface and with visualization of fimbrial structures that could be responsible for the adherence process (Andrade et al. 2011). Although a great diversity of adhesins, toxins, and proteins involved in EAEC pathogenesis has been described, the prevalence of these virulence factors-encoding genes is highly variable and none of these have been found present in all EAEC strains (Czeczulin et al. 1999; Jenkins et al. 2006).

1.6.1 Adherence

Adhesion to the intestinal epithelium is facilitated by fimbriae and is the first step in the bacterial colonization of the gut. Several authors demonstrated that the AA pattern is associated with the presence of fimbrial and afimbrial adhesins in EAEC strains (Bhargava et al. 2009; Boisen et al. 2008; Czeczulin et al. 1997; Hicks et al. 1996). However, the genes encoding these adhesion factors are found in low prevalence, indicating a high diversity of adhesive structures responsible for the AA pattern. The fimbriae bind to components of the extracellular matrix of intestinal epithelial cells (Farfan et al. 2008; Izquierdo et al. 2014b), and the AA pattern is thought to emerge from binding to the epithelial cell surface and binding to adjacent EAEC bacteria.

Abundant adherence of EAEC to the intestinal mucosa includes mucoid biofilm formation. Biofilm formation is a potential important contributor in persistent infection by allowing the bacteria population to evade the local immune system and by

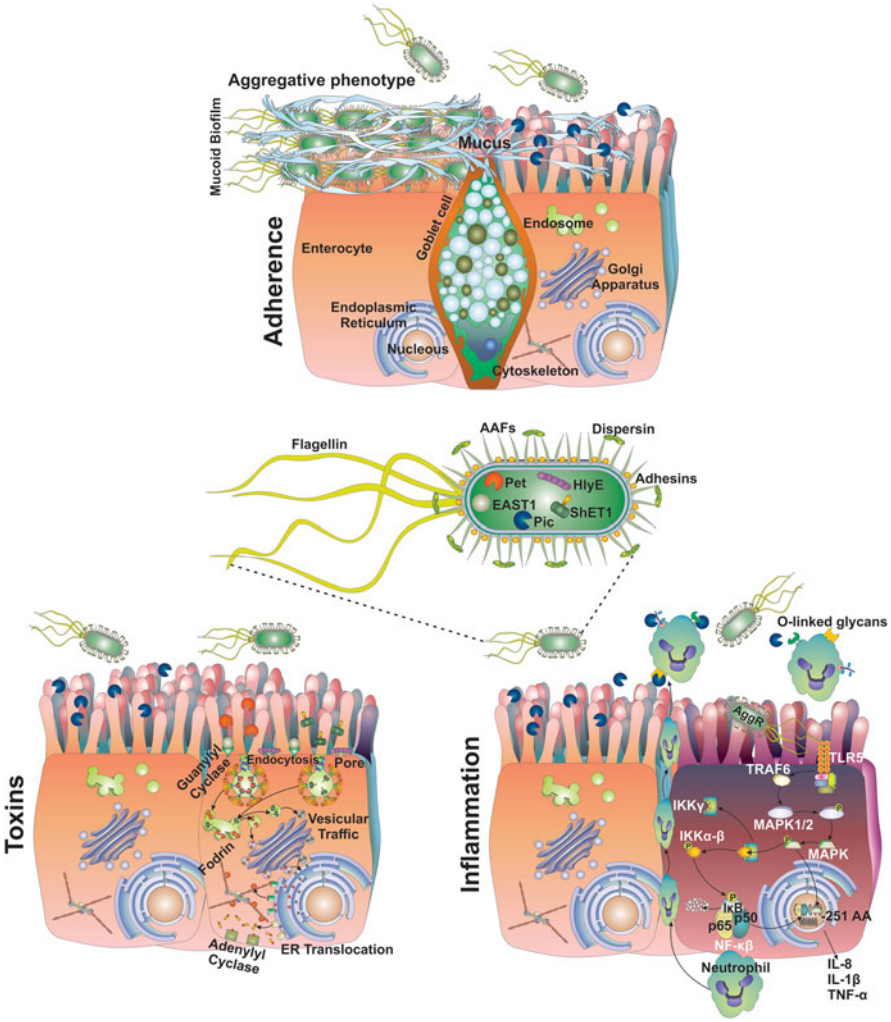

Fig. 2.2 The 3-steps model of EAEC pathogenesis. Three major features of EAEC pathogenesis: (1) abundant adherence to the intestinal mucosa, (2) elaboration of enterotoxins and cytotoxins, and (3) induction of mucosal inflammation

preventing the transport of antibacterial factors, including antibiotics. Secretion of excessive mucus has been described when the gut is colonized with EAEC and this event is followed by the formation of biofilm (Hicks et al. 1996; Navarro-Garcia et al. 2010). Biofilm formation is an important pathogenicity trait of EAEC and it is present mainly in the colon and in the small intestine (Hicks et al. 1996).

1.6.2 Toxins

Once the biofilm has been established, EAEC produces cytotoxic effects such as microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion (Harrington et al. 2006). It is thought that the secretion of toxins plays an important role in secretory diarrhea, which is a typical clinical manifestation of EAEC infection. Numerous putative EAEC virulence factors, such as Pet, EAST-1 and ShET1 toxins, and Pic, have been associated to these cytotoxic effects.

1.6.3 Inflammation

EAEC is an inflammatory pathogen, as demonstrated both in clinical (Greenberg et al. 2002) and laboratory (Steiner et al. 1998) studies. It is clear that multiple factors contribute to EAEC-induced inflammation, and further characterization of the nature of EAEC pro-inflammatory factors will greatly advance the understanding of this emerging pathogen. The initial host inflammatory response to EAEC infection is dependent on the host innate immune system and the type of EAEC strain causing infection. The role of putative virulence genes and clinical outcomes is not well understood; however, the presence of several EAEC virulence factors correlate with findings indicating that levels of fecal cytokines and inflammatory markers in stools of adults and children with diarrhea are elevated, including interleukin (IL)-1 receptor agonist, IL-1β, IL-8, interferon (INF)-γ, lactoferrin, fecal leukocytes, and occult blood (Jiang et al. 2002). The IL-8 inflammatory response appears to be partially caused by flagella (FliC) in a Caco-2 cell assay, as it was found that an aflagellated mutant of EAEC did not produce the same inflammatory response (Steiner et al. 2000).

Besides the pathogenic EAEC mechanisms, host factors are also determinants of EAEC inflammation. It was found that single nucleotide polymorphisms in the promoter region of the gene encoding the lipopolysaccharide receptor CD14 are associated with bacterial diarrhea in US and Canadian travelers to Mexico (Mohamed et al. 2011). The CD14 gene encodes a crucial step in the inflammatory response to bacterial lipopolysaccharide stimulation mediated by the innate immune system. Thus, this study found that one SNP in the promoter region of the CD14 gene was associated with an increased risk of EAEC-induced diarrhea. Patients with the CD14 – 159 TT genotype were significantly associated with EAEC-induced diarrhea compared with healthy controls.

1.7 Main Virulence Factors

In the initial stage of EAEC colonization, the role of fimbrial and afimbrial adhesins is fundamental. Five aggregative adherence fimbriae (AAF/I–AAF/V) have been well characterized in EAEC (Boisen et al. 2008; Czeczulin et al. 1997; Nataro et al. 1992; Jonsson et al. 2015). All AAF fimbriae are encoded by genes located in the pAA

plasmids, which regulated by AggR and their biogenesis follows the usher-chaperone pathway. Also, a type IV fimbrial, called Pil pilus, is responsible for the AA pattern in cultured epithelial cells and abiotic surface and it's exhibited by an atypical EAEC strain isolated in the outbreak in Serbia (Cobeljic et al. 1996; Dudley et al. 2006a).

Non-fimbrial adhesins, or outer membrane proteins with molecular weight between 30 and 58 kDa, and associated with AA pattern have been described in various EAEC strains (Bhargava et al. 2009). In addition, Hra1 and Hra2 are heat-resistant agglutinins involved in autoaggregation, biofilm formation, and aggregative adherence phenotypes (Bhargava et al. 2009; Mancini et al. 2011). The presence of *agn43* gene, encoding the autotransporter protein antigen 43, was associated to biofilm formation and autoaggregation in EAEC 042 (Chaudhuri et al. 2010). The multifactorial characteristic of the AA phenotype is clear from studies with strains expressing multiple adhesins. Furthermore, the low prevalence of genes encoding these adhesins highlights the great diversity of adhesive structures in EAEC.

The dispersin protein (anti-aggregation protein) is an important EAEC virulence factor that mediates the dispersion of EAEC along the intestinal mucosa (Sheikh et al. 2002). Dispersin neutralizes the negative charge on the surface of the bacterial cell and allows AAF/II fimbrial projection, leading to anti-aggregation and dispersal of bacteria in the intestinal wall. This protein requires an ABC-type transport system encoded by the *aatPABCD* operon, which is present in the pAA2 plasmid (Nishi et al. 2003).

As described before, EAEC produces cytotoxic effects evident during in vitro and in vivo studies. Several putative virulence factors associated to those cytotoxic effects have been identified and characterized in EAEC.

The first toxin described in EAEC was the enteroaggregative heat-stable toxin 1 (EAST-1), which is related to the heat-stable toxin (STa) of enterotoxigenic *E. coli* (Savarino et al. 1991). EAST-1 activates adenylate cyclase inducing increased cyclic GMP levels in enterocytes, generating a secretory response (Savarino et al. 1991). EAST-1 is a 38 amino acids peptide (4.1 kDa) encoded by *astA* gene, which is located in pAA2 of EAEC 042 (Czeczulin et al. 1999). Since STa toxin causes secretory diarrhea, it was believed que EAST-1 was responsible for this effects in EAEC-induced diarrhea. However, the presence of EAST-1 in EAEC 17-2 was not sufficient to provoke diarrhea in volunteers (Nataro et al. 1995).

Another toxin encoded by a chromosomal gene in EAEC 042 is called ShET1 (*Shigella* enterotoxin 1), which is encoded by *setAB* located in the antisense strand of *pic* (Henderson et al. 1999a; Navarro-Garcia and Elias 2011). ShET1 is an A:B type toxin that causes accumulation of fluid in rabbit ileal loops. The enterotoxic mechanism of ShET1 is independent of cAMP, cGMP, and calcium. However, the precise mechanism of ShET1 action remains unclear.

Amongst the virulence factors produced by EAEC, the autotransporter proteins have a relevant role in the EAEC pathogenesis. Initially, two autotransporter proteins were identified in EAEC, comprising high-molecular weight proteins of 104 kDa for Pet and 109 kDa for Pic (Eslava et al. 1998; Henderson et al. 1999a). Autotransporter proteins are currently assigned to the type 5 secretion system (T5SS), which is described in detail in Chap. 10. Autotransporters contain three

functional domains: the signal sequence, the passenger domain, and the translocation unit. The signal sequence is present at the N-terminal end of the protein and allows targeting of the protein to the inner membrane for its further export into the periplasm (Jose and Meyer 2007). The passenger domain confers the diverse effector functions. The translocation unit (also called the β-domain), located at the C-terminal end of the protein, consists of a short linker region with an α-helical secondary structure and a β-core that adopts a β-barrel tertiary structure when embedded in the outer membrane and facilitates translocation of the passenger domain (Jose and Meyer 2007).

The serine protease autotransporters of *Enterobacteriaceae* (SPATE) members constitute a group of exoproteins secreted through the T5SS by pathogenic enteric bacteria of the γ-proteobacteria. The passenger domains contain the protease motif (GDSGSP) characteristic of all proteins of the SPATE group (Navarro-Garcia and Elias 2011). SPATEs have been divided into class-1 and class-2 based on structural differences and biological effects; the class-1 SPATEs are related to cytotoxic effects on cultured cells, whereas most class-2 SPATEs exhibit a lectin-like activity with preference to degrade a variety of mucins (Ruiz-Perez and Nataro 2014).

The plasmid and chromosomal EAEC SPATEs Pet and Pic are members of class-1 and class-2, respectively. Originally, Pet and Pic were detected in an effort to identify cytotoxins and enterotoxins secreted by EAEC. Analyses of culture supernatants from strains that caused outbreaks of EAEC diarrhea in Mexican hospitals showed two major proteins of 104 and 109 kDa, which are now known as the secreted proteins Pet and Pic, respectively (Eslava et al. 1998; Henderson et al. 1999a; Navarro-Garcia et al. 1998). Interestingly, both proteins are related with pathogenic features in the infection by EAEC: cytotoxicity and mucosal colonization, including the bacteria-mucus biofilm.

It has been shown that Pet (104 kDa) of EAEC protein is required for inducing dilation of crypt openings and rounding and extrusion of enterocytes in human tissue explants (Henderson et al. 1999b). In an ex-vivo model of infection, Pet causes raises in short-circuit current and decreases in electrical resistance of rat jejunum while mounted in the Ussing chamber, and the enterotoxic effect is accompanied by mucosal damage, increased mucus release, exfoliation of cells, and development of crypt abscesses (Navarro-Garcia et al. 1998). The use of cultured epithelial cells to understand the mode of action of this toxin showed that Pet is a cytoskeleton-altering toxin, because it induces contraction of the cytoskeleton, loss of actin stress fibers, and release of focal contacts followed by complete cell rounding and detachment. Interestingly, Pet cytotoxicity and enterotoxicity depends on Pet serine protease activity (Navarro-Garcia et al. 1999). Furthermore, it was found that Pet requires cytokeratin 8 as receptor to enter the eukaryotic cell and that trafficking through the vesicular system appears to also be required for the induction of cytopathic effects (Nava-Acosta and Navarro-Garcia 2013).

Pet is internalized by receptor-mediated endocytosis, through clathrin-coated vesicles, and this internalization pathway was found to be an essential mechanism because its inhibition prevents Pet internalization, and thereby the Pet-induced cytotoxic effect (Nava-Acosta and Navarro-Garcia 2013). An intracellular target,

α-fodrin (αII-spectrin), has been found for Pet. Pet binds and cleaves (between M^{1198} and V^{1199}) epithelial fodrin in vitro and in vivo, causing fodrin redistribution within the cells to form intracellular aggregates as membrane blebs (Canizalez-Roman and Navarro-Garcia 2003). This mechanism appears to be a new system of cellular damage identified for bacterial toxins, which includes the internalization of the protease to finally allow specific α-fodrin degradation to destroy the cell (Canizalez-Roman and Navarro-Garcia 2003). This was the first report showing cleavage of α-fodrin by a bacterial protease.

Pic was identified as a second SPATE member in EAEC. In contrast to Pet, Pic-encoding gene is localized in the EAEC chromosome (Czeczulin et al. 1999; Henderson et al. 1999a). The *pic* gene has a unique characteristic among the autotransporter proteins since ShET1-encoding genes are oppositely oriented and in tandem within the *pic* gene (Henderson et al. 1999a). Functional analyses of Pic have shown that its serine active site is involved in mucinase activity, serum resistance, and hemagglutination. Phenotypes identified for Pic suggest that it is involved in the early stages of pathogenesis and most probably promotes intestinal colonization (Henderson et al. 1999a). It has also been shown that Pic does not damage epithelial cells (Gutierrez-Jimenez et al. 2008).

As we mentioned before, a hallmark of EAEC infection is the formation of mucoid biofilm, which comprises a mucus layer with immersed bacteria in the intestines of patients. Interestingly, an isogenic *pic* mutant was unable to cause mucus hypersecretion, in contrast to the EAEC wild-type strain, which induced hypersecretion of mucus, accompanied by an increase in the number of mucus-containing goblet cells. Site-directed mutagenesis of the serine protease catalytic residue of Pic showed that, unlike the mucinolytic activity, secretagogue activity does not depend on this catalytic serine protease motif (Navarro-Garcia et al. 2010).

Using specific mutants in competition with the wild-type strain, the contribution of several putative EAEC virulence factors to intestinal colonization of streptomycin-treated mice was evaluated (Harrington et al. 2009). The data suggest that the dispersin surface protein and Pic promote colonization of the mouse. Interestingly, it has been found that Pic targets a broad range of human leukocyte adhesion proteins, such as CD43, CD44, CD45, CD93, CD162, and the surface-attached chemokine fractalkine, all implicated in leukocyte trafficking, migration, and inflammation (Ruiz-Perez et al. 2011). The ability of Pic to inactivate key proteins of all complement pathways is also a new finding, demonstrating its function in immune evasion. Pic significantly reduces complement activation by cleaving C2, C3, C3b, and C4. This is an important virulence mechanism in the context of systemic disease, such as sepsis and hemolytic uremic syndrome, which may be caused by Pic-producing *E. coli* and *Shigella flexneri* (Abreu et al. 2016). All these data strongly suggest that Pic represents a unique immune-modulating bacterial virulence factor.

Besides Pic, another SPATE from class-2 and found in the hybrid EAEC/STEC O104:H4 is SepA (Munera et al. 2014). Several SepA-hydrolyzed peptides were described as specific substrates for cathepsin G, a serine protease produced by polymorphonuclear leukocytes that was proposed to play a role in inflammation. However, unlike cathepsin G, SepA degraded neither fibronectin nor angiotensin

I and had no effect on the aggregation of human platelets. It was found that the gene encoding the autotransporter protease SepA was most strongly associated with diarrhea among the EAEC strains as part of a case-control study of moderate to severe acute diarrhea among children in Mali (Boisen et al. 2012).

SigA is another SPATE of class-1 also detected in EAEC/STEC O104:H4. An initial report showed that the *she* PAI, which contains the *pic* (*she*) gene, also contains a gene encoding a second IgA protease-like homologue, *sigA*. Functional analysis showed that SigA is a secreted temperature-regulated serine protease capable of degrading casein. Performing similar experiments to those used with Pet revealed that SigA is cytopathic for HEp-2 cells, suggesting that it may be a cell-altering toxin with a role in the pathogenesis of *Shigella* infections. Indeed, it was found that SigA binds specifically to HEp-2 cells and degrades recombinant human αII spectrin (α-fodrin) in vitro and also cleaves intracellular fodrin in situ, causing its redistribution within cells (Al-Hasani et al. 2009).

Some pathogenicity islands (PAI) were identified in 042 EAEC strain harboring distinct putative virulence factors (Chaudhuri et al. 2010). The presence of such mobile genetic elements emphasizes the characteristic mosaic of the EAEC genome (Chattaway et al. 2014b). The EAEC 042 *pheU* PAI encodes an important virulence component, the type 6 secretion system (T6SS), extensively studied in many pathogens in the last years. Type 6 secretion system in EAEC 042 is encoded by the *aai* cluster (AggR-activated island), which is composed of 25 genes (Dudley et al. 2006b). In EAEC 042, the T6SS secretes AaiC (Dudley et al. 2006b). The role of the T6SS in the pathogenesis of EAEC has not been established. Also, the genes encoding ShET1 (*setAB*) and the mucinase Pic (*pic*) are contained in a PAI called *she*, which was initially identified in *Shigella* (Henderson et al. 1999a).

Analysis of the genome of EAEC strain 042 indicated the presence of genes encoding a type 3 secretion system at *glyU* tRNA locus (Chaudhuri et al. 2010), which in other pathogens is associated with the translocation of effectors proteins directly into the cytoplasm of eukaryotic cells (see Chap. 10). This system is named ETT2 (*Escherichia coli* type 3 secretion system 2) and presents high similarity to that found in enterohemorrhagic *E. coli* and *Salmonella*. The possible ETT2 effectors are located at *selC* tRNA locus, which harbors the *eipBCD*, *eicA,* and *air* genes. The bacterial surface protein Air is involved in adherence and aggregation of EAEC 042 (Sheikh et al. 2006). The functionality of ETT2 and the role of possible translocated effectors in EAEC pathogenesis remain elusive.

1.8 Diagnostic

The AA pattern demonstrated in adhesion assays using HeLa or HEp-2 cells is still considered the gold standard test for the identification of EAEC since a common genetic determinant for all strains of this pathotype has not yet found (Hebbelstrup Jensen et al. 2014). However, this technique is costly, time consuming, and requires infrastructure restricted to reference laboratories. Therefore, molecular diagnostic

techniques have been developed as an alternative to detection of the AA pattern, which started with the development of a DNA probe (CVD432), initially described as a cryptic fragment of the pAA1 plasmid of EAEC strain 17-2. This probe has been widely used in diagnosis and epidemiological studies showing high specificity but variable sensitivity (20–89 %) when compared to the detection of the AA pattern. Later, CVD432 was characterized as part of the *aatA* gene, which is part of the dispersin-secretion system operon (Nishi et al. 2003). Thus, CVD432 probe is properly called *aatA* or AA probe. As *aatA* is a pAA-borne gene, its detection excludes the majority of atypical EAEC.

There are several reports in the literature on the development of multiplex PCR for simultaneously detection of all diarrheagenic *E. coli* pathotypes. However, those detecting EAEC use only one or two pAA markers, excluding the detection of atypical EAEC (Panchalingam et al. 2012). Some authors have proposed the diagnosis of EAEC only based on multiplex PCR, detecting either the plasmid or plasmid and chromosomal markers (Andrade et al. 2014a). Recently, the detection of *aatA aggR* and *aaiG/aaiC* has been proposed as an alternative for sensitive and specific molecular detection of EAEC, covering the detection of both typical and atypical subgroups (Andrade et al. 2014a; Boisen et al. 2012; Panchalingam et al. 2012).

1.9 Transmission

There is no evidence for an animal reservoir of EAEC; therefore, humans are generally accepted as the reservoir. The transmission of EAEC is often associated with food-borne sources or by contaminated water (Jiang et al. 2002). Food-borne outbreaks have been described (Itoh et al. 1997). Risk factors for EAEC infection include travel to developing countries, ingestion of contaminated food and water, poor hygiene, host susceptibility, and possibly immunosuppression (Estrada-Garcia and Navarro-Garcia 2012). Another route of transmission of EAEC is food handling. A study of Mexican tabletop sauces identified 44 % of sauces containing viable EAEC (Adachi et al. 2002).

1.10 Treatment

The oral rehydration therapy is the prime recommendation for the cases of self-limited acute diarrhea caused by EAEC. Antibiotic therapy is indicated only in cases of persistent diarrhea, and should be considered case by case. When required, antimicrobial sensitivity evaluation tests are indicated since multidrug resistance has been often reported in EAEC strains isolated from patients from different geographic regions (Hebbelstrup Jensen et al. 2014). Alternative treatments, employing the use of zinc and nitazoxanide, have been recently proposed for treatment and prevention of diarrhea based on clinical and laboratorial evidences (Bolick et al. 2013).

2 Recent Advances

2.1 Intestinal Inflammation

Several lines of evidence suggest that EAEC infection is mildly inflammatory in nature. Epidemiological reports have documented elevated fecal lactoferrin, IL-8, and IL-1β among infected infants in developing countries and adult travelers to India and Mexico (Steiner et al. 1998; Greenberg et al. 2002). Travelers to Mexico who developed symptomatic illness due to EAEC infection excreted high concentrations of fecal IL-8, compared to travelers who did not develop diarrhea due to EAEC infection (Jiang et al. 2003). Steiner and colleagues have shown that EAEC strain 042 induces IL-8 release from non-polarized Caco-2 intestinal epithelial cells (IECs); the pAA plasmid was required for the full inflammatory effect (Steiner et al. 1998). These investigators also reported that a mutation in the gene encoding flagellin (*fliC*) abrogated IL-8 release, implicating flagellin as the major pro-inflammatory stimulus (Steiner et al. 2000). However, it has been reported that significantly more IL-8 was detected in feces of travelers infected with EAEC strains harboring the plasmid-borne *aggR* or *aafA* genes, compared with those infected with virulence factor-negative EAEC (Jiang et al. 2002). In a search to identify additional factors that could account for the IL-8 release from epithelial cells infected with EAEC strain 042, it was found that polarized T84 intestinal cells release IL-8, even when infected with 042 mutated in the major flagellar subunit FliC. IL-8 release from polarized T84 cells was found to require the AggR activator and the AAF fimbriae, and IL-8 release was significantly less when cells were infected with mutants in AafB, the minor pilin subunit of AAF/II (Harrington et al. 2005). In addition to IL-8, intestinal epithelial cells infected with EAEC 042 have been shown to upregulate the following genes: IL-6, tumor necrosis factor (TNF)-α, growth-related gene product (GRO)-α, GRO-γ, intercellular adhesion molecule (ICAM)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-1α. These cellular responses are primarily mediated by flagellin (FliC) of EAEC (Harrington et al. 2005). It is clear that multiple factors contribute to EAEC-induced inflammation, and further characterization of the nature of EAEC pro-inflammatory factors will greatly advance the understanding of this emerging pathogen.

2.2 Growth Impairment in Developing Countries

EAEC persistent diarrhea has been linked to malnutrition and decreased physical and cognitive development in children (Guerrant et al. 2008). Interestingly, in this study the population, even asymptomatic patients infected with EAEC, exhibited growth retardation compared to uninfected controls (Steiner et al. 1998). Thus, it seems that EAEC effects on growth shortfalls in children could also be due to its

persistence in human intestine, with subclinical symptoms, inducing chronic inflammation in the absence of diarrheal disease (Steiner et al. 1998). Pathogenic EAEC infection is characterized by release of cytokines from the intestinal mucosa and lactoferrin, revealing the inflammatory potential of these strains in damaging the intestinal epithelium and reducing its absorptive function, leading to nutrient depletion and malnutrition. In turn, malnutrition further facilitates infection and perpetuates the cycle of infection, malnutrition, and developmental deficits, increasing the burden of the disease (Guerrant et al. 2008). Given the high rate of asymptomatic excretion of EAEC in a large number of countries in the developing world, understanding its potential role in malnutrition and growth retardation is a high priority.

2.3 Animal Models for EAEC Pathogenesis

Researchers have been trying to develop animal models to reproduce human diarrhea and immunological responses at the site of EAEC infection. Development of such models could allow the evaluation of pathophysiology, treatment, and prototype vaccines.

Adult CD-1, Balb/c, and C3H/HeJ mice, treated with streptomycin, have been used to study intestinal colonization (Harrington et al. 2009). These murine models are suitable to quantify intestinal colonization, verify histological alterations, and determine immunological mediators in fecal contents although mice do not develop enteritis symptoms. Using these models, Harrington and colleagues demonstrated the role of autotransporter Pic in promoting mucus secretion and colonization (Harrington et al. 2009).

A murine model to assess EAEC infection malnutrition cycle was developed using neonatal and weaned C57BL/6 mice (Roche et al. 2010). Although mild histological effects in the colonic epithelium were seen, growth impairment could be assessed in both groups and in weaned mice, infection was persistent with stool shedding. Undernutrition in both groups of mice intensified infection and growth impairment was dependent on bacterial burden and challenge dose. However, this model has some limitations, due to the early age of the animals. For that reason, the same approach has been applied to infect nourished and malnourished weaned 24-day-old C57Bl/6 mice and similar results were obtained (Bolick et al. 2013). This model was adapted to investigate the role of zinc in preventing EAEC pathogenesis. Weight loss, stool shedding, mucus production and, more importantly, diarrhea, were observed when mice fed a zinc-free and protein source defined diet, pretreated with antibiotics and then orally challenged with EAEC 042.

A novel model for studying EAEC disease has been developed with human fetal intestinal xenografts in severe-combined immunodeficient (SCID) mice (Boll et al. 2012). The successful transplants allowed the study of intact and functional human intestinal tissue infected with EAEC 042, showing severe tissue damage and infiltration of polymorphonuclear cells, with pathogenesis strongly related to AAF/II

expression. This model can address the investigation of the role of specific virulence factors and the interaction of EAEC with human intestinal mucosa. However, some limitations of the model include the restriction to fetal human tissue, the lack of intestinal microbiota in the xenografts, and interaction of innate and adaptive immunity.

2.4 Urinary Tract Infection

In the last years, a growing number of reports have shown a linkage between EAEC and urinary tract infection (UTI). Some studies investigated the presence of typical virulence factors of EAEC in strains causing extraintestinal infections (Abe et al. 2008; Herzog et al. 2014). Others demonstrated the presence of uropathogenic *E. coli* (UPEC) markers in EAEC collections (Chattaway et al. 2014a). These findings clearly indicate the potential for some EAEC strains to cause UTI.

Indeed, Olesen and colleagues reported a community-acquired UTI outbreak caused by EAEC (Olesen et al. 2012), which does not fulfill the extraintestinal *E. coli* (ExPEC) criteria. This multiresistant strain was characterized as belonging to the serotype O78:H10, multilocus sequence type ST10, and the phylogenetic group A (Olesen et al. 2012). This was the first time that EAEC was implicated as an agent of an outbreak of extraintestinal disease. Subsequently, the uropathogenic properties of this EAEC O78:H10 strain were investigated showing that these properties were conferred by specific virulence factors of the EAEC, such as the AAF/I fimbria (Boll et al. 2013). Recently, EAEC was also implicated as a causative agent of one case of urosepsis (Herzog et al. 2014).

2.5 The Hybrid O104:H4 STEC/EAEC Strain

An outbreak caused by *E. coli* of serotype O104:H4 spread throughout Germany in May 2011 (Navarro-Garcia 2014). This was the largest outbreak by a pathogenic *E. coli* strain, with 3128 cases of acute gastroenteritis, 782 cases of hemolytic uremic syndrome (HUS), and 46 deaths. All these cases were officially attributed to a new clone of STEC and most of the victims became infected in Germany or France. The phenotypic and genotypic characterization of the *E. coli* O104:H4 indicated that the isolates from the French and German outbreaks were common to both incidents. Fenugreek seeds imported from Egypt, from which sprouts were grown, were implicated as a common source. However, there is still much uncertainty about whether this is truly the common cause of the infections, as tests on the seeds did not allow the detection of any *E. coli* isolate of serotype O104:H4.

This large outbreak was caused by an unusual STEC strain, which is more similar to an EAEC of serotype O104:H4. A significant difference, however, is the presence of a prophage encoding the Shiga toxin, which is characteristic of STEC strains

(Rasko et al. 2011). This unique combination of genomic features, associating characteristics from both EAEC and STEC, represents a new pathotype. Since typical EAEC is isolated primarily from humans, the origin of this outbreak may not be zoonotic.

Due to the special features and high pathogenicity of this hybrid clone, one provocative question is what makes this *E. coli* O104:H4 outbreak strain so dangerous. One explanation is that this hybrid strain with EAEC features but with a phage coding for Shiga toxin type 2 is a better colonizer of the gut due to its aggregative phenotype. The enhanced adherence of this strain to intestinal epithelial cells might facilitate systemic absorption of Shiga toxin and could explain the high frequency of cases progressing to HUS. Thereby, it is believed that EAEC of serotype O104:H4 is by itself an emerging serovar that has acquired an original set of virulence factors (Frank et al. 2011).

EAEC strains of serotype O104:H4 contain a large set of virulence-associated genes regulated by AggR. These include the pAA plasmid genes encoding the AAF, which anchor the bacterium to the intestinal mucosa and induce inflammation, as well as a protein-coat secretion system (Aat), that secretes the protein dispersin (Navarro-Garcia 2014). Thus, a switch of pAA together with the type of the aggregative adherence fimbriae could be an additional explanation for the higher virulence of this outbreak strain. Indeed, the outbreak STEC O104:H4 strain is similar to the EAEC O104:H4 strain 55989, isolated in the late 1990s, from a patient in the Central African Republic with persistent diarrhea, and to the EAEC O104:H4 strain HUSEC041 that was associated in 2001 with very few HUS cases in Germany. Interestingly, the EAEC O104:H4 strain HUSEC041 carries the plasmid encoding AAF/III fimbriae (also present in the EAEC strain 55989). In contrast, outbreak EAEC O104:H4 isolates acquired a new plasmid, encoding AAF/I fimbriae, and lost the plasmid encoding AAF/III fimbriae (Rasko et al. 2011).

Other interesting features that might also contribute to the higher virulence of this outbreak strain include that EAEC strains of serotype O104:H4 produce a variable number of SPATEs implicated in mucosal damage and colonization. This new serovar contains Pic, SigA, and SepA (Rasko et al. 2011). Rasko and colleagues speculate that the combined activity of these SPATEs together with other EAEC virulence factors accounts for the increased uptake of Shiga toxin into the systemic circulation, resulting in the high rates of HUS (Rasko et al. 2011). Indeed, a recent study showed that SPATEs but not pAA are critical for rabbit colonization by Shiga toxin-producing *E. coli* O104:H4 (Munera et al. 2014). However, it has been also shown recently that the presence of the pAA plasmid in the EAEC/STEC O104:H4 strain promotes the translocation of Stx2a across an epithelial cell monolayer (Boisen et al. 2014). Interestingly, Ec55989 contains three copies of *pic*, which are conserved in the German outbreak strain. In addition, there is a fourth *pic* gene present in the EAEC plasmid of the outbreak strain. The outbreak strain also encodes SigA that cleaves the cytoskeletal protein spectrin, inducing rounding and exfoliation of enterocytes (Al-Hasani et al. 2009), and SepA, associated with increased *S. flexneri* virulence, but with unknown function in EAEC.

The ability of STEC to cause severe disease in humans is mainly associated with the production of one of the two Shiga toxin groups, Stx1 and Stx2, with similar biological activity but different immunogenicity. The EAEC/STEC hybrid

clone produces the Shiga toxin 2 (Stx2). An interesting finding that highlights Stx2 is its epidemiological association with severe diseases in humans (Friedrich et al. 2002).

Two remarkable features of EAEC O104:H4 have to be highlighted: it is the agent of a massive outbreak and the high proportion of cases developing HUS. This complication was diagnosed in a 22 % of the cases, while historical rates of HUS after O157:H7 infection typically range from 6 to 15 % (Frank et al. 2011). It is challenging to know why the outbreak strains are so virulent, given that EAEC are human-specific pathogens and that few animal infection models mimic human disease. Interestingly, the lack of the pAA plasmid abolished the capacity of an outbreak strain (C227-11) to adhere to viable colonic tissue harvested from the cynomolgus monkey *Macaca fascicularis* (Boisen et al. 2014). However, C227-11 adhered in an aggregative manner and forms heavy biofilms with a thick mucus layer upon interaction with the monkey colonic tissue.

2.6 Vaccine Development

A few studies in the last years tried to use aggregative adherence fimbriae (AAF) as candidate vaccine antigens. AAF/I and AAF/II structural genes (*aafA* and *aggA*, respectively) were coupled to the gene encoding nontoxic B subunit of Shiga toxin (StxB). The recombinant polypeptides elicited immune response in subcutaneously immunized Balb/C, and the antibodies generated inhibited the adherence of prototype EAEC strains to HeLa cells and protected the immunized mice against a lethal dose of Shiga toxin (Oloomi et al. 2009).

The use of the polysaccharide part of LPS has been evaluated as a vaccine target against EAEC belonging to the serogroup O111. However, LPS is not appropriate for human use due to its high level of toxicity. As an alternative, polysaccharide of O111 LPS was conjugated with cytochrome C or recombinant B subunit of the heat-labile toxin (LT). These two different approaches induced systemic and mucosal antibodies in rabbit and in mice, which were able to inhibit the adhesion of all categories of O111 *E. coli* to HEp-2 cells serogroups (Andrade et al. 2014b). Although, this approach protects against one O-antigen, it does not represent the vast diversity of circulating EAEC serogroups.

A combined formalin-killed whole-cell vaccine candidate, consisting of a mixture of EAEC (strain 17-2), EPEC, STEC, enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC), was also proposed as vaccine (Gohar et al. 2016). Balb/C mice were immunized subcutaneously, eliciting humoral immune response to each pathotype. In addition, the specific antibodies were protective when mice were challenged intraperitoneally with the respective immunizing bacteria. Although, such approach is based in a subcutaneous immunization, which is not common in endemic areas, and only antibodies against EAEC 17-2 were generated.

3 Advances on EAEC in the Americas

3.1 Recent Epidemiological Information

A few studies about the etiology of diarrhea in the Americas have been published between 2013 and March 2016, including children and adults hospitalized with diarrhea or with diarrhea in the community (Table 2.2). The studies that searched only for viruses and/or parasitic enteropathogens were not included. When searched in the studies, EAEC strains were found as the most prevalent diarrheagenic *E. coli* pathotype.

In Mexico, Patzi-Vargas and colleagues determined the prevalence of bacterial enteropathogens in 831 children with acute diarrhea (Patzi-Vargas et al. 2015). Diarrheagenic *E. coli* was the most common bacterial enteropathogen and the predominant pathotypes were diffusely adherent *E. coli* (35 %) and EAEC (24 %). EAEC was more frequent in children between 6 and 24 months old than in those younger than 6 months of age. In addition, all diarrheagenic *E. coli* strains were searched for supplementary virulence genes (SVG) mainly associated with EAEC. Dispersin (*aap*), dispersin-translocator (*aatA*), EAST-1 (*astA*), plasmid-encoded toxin (*pet*), and cytolethal distending toxin (*cdt*) were higher in diarrheagenic *E. coli* than non-diarrheagenic *E. coli* strains and 98 % of EAEC-infected children harbored strains with SVG; 85 % carried the *aap-aatA* gene combination and 33 % of these carried *astA*. Diarrheagenic *E. coli* carrying SVG was a cause of moderate to severe bacterial diarrhea in that population.

EAEC was found in 13 % of rural Panamanian children (Jimenez Gutierrez et al. 2014). In Brazil, coinfections of EAEC and norovirus were detected in 24.1 % of fecal samples from hospitalized diarrheic children (Amaral et al. 2015). In another study from Brazil, EAEC was the most frequent pathotype found in cases and controls (10 %) although without statistical differences between the two groups (Dias et al. 2016). A large study in Bolivia (3943 cases and 1026 controls) showed that EAEC was the most prevalent pathotype found in 11.2 % of the children (Gonzales et al. 2013).

3.2 Adhesins

Advances in the comprehension of EAEC binding mechanisms to the human intestinal tissue have been achieved by characterizing the structure, binding characteristics, and immunogenicity of AAF fimbriae. These approaches are necessary in order to develop efficient blocking strategies. Since AAF/II is the main adhesive structure of EAEC 042, much of the data is based on recent studies characterizing receptors for its pilin and its molecular structure.

Adhesion of EAEC 042 to extracellular matrix proteins (fibronectin, laminin, and type IV collagen) was demonstrated in vitro. Also, purified AafA bound fibronectin in a dose-dependent manner (Farfan et al. 2008). Since the major cellular

Table 2.2 Epidemiological studies on the etiology of acute diarrhea that included the detection of EAEC in American countries (2013–2016)

Country	Study population	Number of patients	Diarrheagenic *E. coli* pathotypes detected (%)	References
Bolivia	Hospitalized children with acute diarrhea <5 years old	3943 cases and 1026 controls	Cases: EAEC (11.2 %), ETEC (6.6 %), EPEC (5.8 %)	Gonzales et al. (2013)
			Controls: EAEC (7.4 %), ETEC (4.8 %), EPEC (4 %)	
Brazil	Adults and children (outbreaks, serious cases of diarrhea, sporadic diarrhea)	400 cases	EAEC (0.7 %), aEPEC (3.2 %), DAEC (0.7 %), tEPEC (0.2 %), STEC (0.2 %)	Assis et al. (2014)
Brazil	Hospitalized children with acute diarrhea <6 years old	63 cases with viral agents of diarrhea	EAEC (15.9 %), aEPEC (6.3 %), tEPEC (3.2 %), EHEC (1.6 %)	Amaral et al. (2015)
Brazil	Children <5 years old	200 cases and 200 controls	Cases: EAEC (10 %), aEPEC (8 %), ETEC (0.5 %), STEC (0.5 %)	Dias et al. (2016)
			Controls: EAEC (10 %), aEPEC (8.5 %), tEPEC (0.5 %), ETEC (0.5 %), STEC (0.5 %)	
Multicenter study (data from Brazil)	Group 1: 0–11 months	129 diarrhea episode stools and 2519 surveillance stools	Cases 0–11 months: EAEC (34.2 %), ETEC (21.1 %), aEPEC (7.9 %), tEPEC (7.9 %), EIEC (5.3 %)	Platts-Mills et al. (2015)
			Controls 0–11 months: EAEC (37.8 %), aEPEC (13.3 %), EIEC (12.7 %), ETEC (5.9 %), tEPEC (5.8 %)	
	Group 2: 12–24 months (community surveillance)		Cases 12–24 months: EAEC (34.2 %), ETEC (12.7 %), EIEC (12.7 %), aEPEC (11.4 %), tEPEC (10.1 %)	
			Controls 12–24 months: EAEC (41.9 %), EIEC (16.7 %), aEPEC (13.1 %), ETEC (9.9 %), tEPEC (6.9 %)	
Multicenter study (data from Peru)	Group 1: 0–11 months	2047 diarrhea episode stools and 3185 surveillance stools	Cases 0–11 months: EAEC (15.1 %), ETEC (5.7 %), aEPEC (4 %)	Platts-Mills et al. (2015)
			Controls 0–11 months: EAEC (16.1 %), aEPEC (4.8 %), ETEC (4.4 %)	
	Group 2: 12–24 months (community surveillance)		Cases 12–24 months: EAEC (18.9 %), ETEC (7.6 %), aEPEC (7.2 %)	
			Controls 12–24 months: EAEC (20.2 %), aEPEC (7.3 %), ETEC (7.1 %)	

Colombia	Children <5 years old	466 cases and 349 controls	Cases: EAEC (0.3 %), ETEC (4.9 %), EPEC (1.4 %), EIEC (0.3 %), mixed DEC (0.6 %)	Gomez-Duarte et al. (2013)
			Controls: EAEC (0.9 %), ETEC (2.3 %), EPEC (0.3 %)	
Mexico	Hospitalized children with acute diarrhea <5 years old	831 cases	EAEC (24 %), DAEC (35 %), aEPEC (16 %), ETEC (9 %), tEPEC (4 %), STEC (0.4 %), EIEC (0.4 %), mixed DEC (10 %)	Patzi-Vargas et al. (2015)
Nicaragua	Children <5 years old (households)	337 cases and 106 controls	Cases: EAEC (3.6 %), EPEC (11.3 %), ETEC (7.7 %), EHEC (3 %)	Becker-Dreps et al. (2014)
			Controls: EAEC (4.7 %), EPEC (14.2 %), ETEC (6.6 %), EHEC (0.9 %)	
Panama	Children <5 years old (rural residents)	87 cases	EAEC (12.6 %)	Jimenez Gutierrez et al. (2014)
Uruguay	Children <5 years old (high socioeconomic level, households)	59 cases	EAEC (6.8 %), aEPEC (10.2 %), tEPEC (5.1 %), STEC (5.1 %)	Varela et al. (2015)

EAEC enteroaggregative *E. coli, aEPEC* atypical enteropathogenic *E. coli, tEPEC* typical enteropathogenic *E. coli, ETEC* enterotoxigenic *E. coli, STEC* Shiga toxin-producing *E. coli, EHEC* enterohemorrhagic *E. coli, EIEC* enteroinvasive *E. coli, DAEC* diffusely adherent *E. coli, DEC* diarrheagenic *E. coli*

receptor of fibronectin is integrin α5β1, Izquierdo and colleagues evaluated the participation of this receptor in the fibronectin-mediated adherence of EAEC strain 042 to intestinal cells (Izquierdo et al. 2014a). They identified the complex AafA/fibronectin/integrin α5β1 and showed that EAEC strain 042 has the ability to bind directly and indirectly to integrin α5β1; the indirect binding is mediated by AAF/II and fibronectin. Subsequent studies confirmed the binding to laminin and showed the involvement of the major subunit of AAF/II fimbriae (AafA) in the binding to cytokeratin 8, indicating a role of CK8 as a potential receptor for EAEC (Izquierdo et al. 2014b).

New atomic resolution insights on the structure of AAFI and AAF/II was achieved by X-ray crystallography and nuclear magnetic resonance structures (Berry et al. 2014). The major pilin subunits (AggA and AafA) assemble into linear polymers by donor strand complementation, where a single minor subunit (AggB and AAfB) is inserted at the tip of the polymer by accepting the donor strand from the terminal major subunit. The minor subunits are conserved while the major subunits display large structural differences. In spite of that, both AAF recognized fibronectin as receptor. All together, these data reinforce the role of fibronectin, cytokeratin 8, and laminin as receptors for AAF.

3.3 Hybrid EAEC/STEC Strain

Rapid genome sequencing and public availability of these data from the EAEC/STEC outbreak strain allowed the identification of an O-antigen-specific bacteriophage tail spike protein encoded in the genome (Scholl et al. 2012). These authors synthesized this gene and fused it to the tail fiber gene of an R-type pyocin, a phage tail-like bacteriocin, and expressed the novel bacteriocin such that the tail fiber fusion was incorporated into the bacteriocin structure. The resulting particles have bactericidal activity specifically against *E. coli* strains that produce the O104 lipopolysaccharide antigen, including the outbreak strain. This O-antigen tail spike-R-type pyocin strategy provides a platform to respond rapidly to emerging pathogens upon the availability of the pathogen's genome sequence (Scholl et al. 2012).

Recently, Carbonari et al. (2014) reported the first isolation of an EAEC O104:H4 strain associated with an acute diarrhea case in Argentina (Carbonari et al. 2014). The identified *E. coli* strain was susceptible to all antimicrobials tested and harbored the *aggR*, *aaiC*, pAA plasmid, *lpfO113*, *rfbO104*, *fliCH4,* and *terD* genes. Although serotype EAEC O104:H4 rarely spreads and sporadic cases have been reported, global concern increased after the large-scale outbreak in Europe in 2011. The finding of EAEC O104:H4 reinforces the need for improved methodologies for the detection of all *E. coli* pathotypes (Carbonari et al. 2014).

More recently, Ross et al. (2015) studied the role of long polar fimbriae (*lpf*) *lpf1* and *lpf2* operons encoded in *E. coli* O104:H4 (Ross et al. 2015). Isogenic *lpfA1* and *lpfA2* major fimbrial subunit mutants were assessed their ability to adhere to intestinal epithelial cells. The Δ*lpfA1* showed decreased adherence in both cell systems while the Δ*lpfA2* only showed a decrease in adherence to polarized Caco-2 cells. Additionally, it was found that the Δ*lpfA1* was unable to form a stable biofilm and in an in vivo murine model of intestinal colonization, the Δ*lpfA1* had a reduced ability to colonize the cecum and large intestine. Further, in competitive assays the presence of the wild-type O104:H4 facilitates increased adherence of the Δ*lpfA1* to levels exceeding that of the wild type in the in vitro and in vivo models. Thus, these data demonstrated that Lpf1 is one of the factors responsible for O104:H4 intestinal adhesion and colonization (Ross et al. 2015).

4 Conclusions

In conclusion, much progress has been made in recent years towards understanding the pathogenesis and epidemiology of EAEC. It has been consistently shown that EAEC is a globally important pathogen, affecting both children and adults, unlike other diarrheagenic *E. coli* pathotypes that are only prevalent in developing countries or agents of sporadic outbreaks. The role of EAEC as an agent of urinary tract infections was also demonstrated recently, indicating its relevance as a pathogen of extraintestinal infections, another peculiar characteristic of EAEC. The ability of a strain of EAEC O104:H4 in acquiring the Stx phage generated a highly virulent pathogen that was responsible for the largest outbreak of diarrhea and HUS reported to date. This hybrid EAEC/STEC was detected in strains of other serotypes but related to small HUS outbreaks or cases of bloody diarrhea. Another advance in the knowledge of the pathogenesis of EAEC was the close relationship between growth and cognitive impairment in malnourished children and EAEC as the agent of repeated cases of diarrhea or even in asymptomatic colonization. New challenges concerning EAEC research reside in the definition of which set of virulence genes define the virulent strains within the complex heterogeneity of this pathotype; the choice of immunogenic antigens with pan-protection coverage for these heterogeneous strains; and the comprehension of all mechanisms and bacterial factors involved in the intestinal inflammation process induced by EAEC.

Acknowledgments The authors were supported by a Conacyt grant (221130) to FNG and by FAPESP and CNPq grants to WPE. We also thank Paul S. Ugalde for the artistic work in Fig. 2.2, Lucia Chavez-Dueñas for organizing the reference database and Fernando H. Martins for his help with collecting epidemiological data in American countries.

References

Abe CM, Salvador FA, Falsetti IN, Vieira MA, Blanco J, Blanco JE, Blanco M, Machado AM, Elias WP, Hernandes RT, Gomes TA (2008) Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunol Med Microbiol 52(3):397–406

Abreu AG, Abe CM, Nunes KO, Moraes CT, Chavez-Duenas L, Navarro-Garcia F, Barbosa AS, Piazza RM, Elias WP (2016) The serine protease Pic as a virulence factor of atypical enteropathogenic *Escherichia coli*. Gut Microbes 7(2):115–125

Adachi JA, Mathewson JJ, Jiang ZD, Ericsson CD, DuPont HL (2002) Enteric pathogens in Mexican sauces of popular restaurants in Guadalajara, Mexico, and Houston, Texas. Ann Intern Med 136(12):884–887

Al-Hasani K, Navarro-Garcia F, Huerta J, Sakellaris H, Adler B (2009) The immunogenic SigA enterotoxin of *Shigella flexneri* 2a binds to HEp-2 cells and induces fodrin redistribution in intoxicated epithelial cells. PLoS One 4(12), e8223

Amaral MS, Estevam GK, Penatti M, Lafontaine R, Lima IC, Spada PK, Gabbay YB, Matos NB (2015) The prevalence of norovirus, astrovirus and adenovirus infections among hospitalised children with acute gastroenteritis in Porto Velho, state of Rondonia, western Brazilian Amazon. Mem Inst Oswaldo Cruz 110(2):215–221

Andrade JA, Freymuller E, Fagundes-Neto U (2011) Adherence of enteroaggregative *Escherichia coli* to the ileal and colonic mucosa: an in vitro study utilizing the scanning electron microscopy. Arq Gastroenterol 48(3):199–204

Andrade FB, Gomes TA, Elias WP (2014a) A sensitive and specific molecular tool for detection of both typical and atypical enteroaggregative *Escherichia coli*. J Microbiol Methods 106:16–18

Andrade GR, New RR, Sant'Anna OA, Williams NA, Alves RC, Pimenta DC, Vigerelli H, Melo BS, Rocha LB, Piazza RM, Mendonca-Previato L, Domingos MO (2014b) A universal polysaccharide conjugated vaccine against O111 *E. coli*. Hum Vaccines Immunother 10(10):2864–2874

Assis FE, Wolf S, Surek M, De Toni F, Souza EM, Pedrosa FO, Farah SM, Picheth G, Fadel-Picheth CM (2014) Impact of Aeromonas and diarrheagenic *Escherichia coli* screening in patients with diarrhea in Parana, southern Brazil. J Infect Dev Ctries 8(12):1609–1614

Baudry B, Savarino SJ, Vial P, Kaper JB, Levine MM (1990) A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. J Infect Dis 161(6):1249–1251

Becker-Dreps S, Bucardo F, Vilchez S, Zambrana LE, Liu L, Weber DJ, Pena R, Barclay L, Vinje J, Hudgens MG, Nordgren J, Svensson L, Morgan DR, Espinoza F, Paniagua M (2014) Etiology of childhood diarrhea after rotavirus vaccine introduction: a prospective, population-based study in Nicaragua. Pediatr Infect Dis J 33(11):1156–1163

Bernier C, Gounon P, Le Bouguenec C (2002) Identification of an aggregative adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as a sensitive probe for detecting the AAF-encoding operon family. Infect Immun 70(8):4302–4311

Berry AA, Yang Y, Pakharukova N, Garnett JA, Lee WC, Cota E, Marchant J, Roy S, Tuittila M, Liu B, Inman KG, Ruiz-Perez F, Mandomando I, Nataro JP, Zavialov AV, Matthews S (2014) Structural insight into host recognition by aggregative adherence fimbriae of enteroaggregative *Escherichia coli*. PLoS Pathog 10(9), e1004404

Bhargava S, Johnson BB, Hwang J, Harris TA, George AS, Muir A, Dorff J, Okeke IN (2009) Heat-resistant agglutinin 1 is an accessory enteroaggregative *Escherichia coli* colonization factor. J Bacteriol 191(15):4934–4942

Boisen N, Struve C, Scheutz F, Krogfelt KA, Nataro JP (2008) New adhesin of enteroaggregative *Escherichia coli* related to the Afa/Dr/AAF family. Infect Immun 76(7):3281–3292

Boisen N, Scheutz F, Rasko DA, Redman JC, Persson S, Simon J, Kotloff KL, Levine MM, Sow S, Tamboura B, Toure A, Malle D, Panchalingam S, Krogfelt KA, Nataro JP (2012) Genomic characterization of enteroaggregative *Escherichia col*i from children in Mali. J Infect Dis 205(3):431–444

Boisen N, Hansen AM, Melton-Celsa AR, Zangari T, Mortensen NP, Kaper JB, O'Brien AD, Nataro JP (2014) The presence of the pAA plasmid in the German O104:H4 Shiga toxin type 2a (Stx2a)-producing enteroaggregative *Escherichia coli* strain promotes the translocation of Stx2a across an epithelial cell monolayer. J Infect Dis 210(12):1909–1919. doi:10.1093/infdis/jiu399

Bolick DT, Roche JK, Hontecillas R, Bassaganya-Riera J, Nataro JP, Guerrant RL (2013) Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. J Med Microbiol 62(Pt 6):896–905

Boll EJ, Struve C, Sander A, Demma Z, Nataro JP, McCormick BA, Krogfelt KA (2012) The fimbriae of enteroaggregative *Escherichia coli* induce epithelial inflammation in vitro and in a human intestinal xenograft model. J Infect Dis 206(5):714–722

Boll EJ, Struve C, Boisen N, Olesen B, Stahlhut SG, Krogfelt KA (2013) Role of enteroaggregative *Escherichia coli* virulence factors in uropathogenesis. Infect Immun 81(4):1164–1171

Canizalez-Roman A, Navarro-Garcia F (2003) Fodrin CaM-binding domain cleavage by Pet from enteroaggregative *Escherichia coli* leads to actin cytoskeletal disruption. Mol Microbiol 48(4):947–958

Carbonari CC, Deza N, Flores M, Gasparini A, Manfredi E, Rivas M (2014) First isolation of enteroaggregative *Escherichia coli* O104:H4 from a diarrhea case in Argentina. Rev Argent Microbiol 46(4):302–306

Chattaway MA, Jenkins C, Ciesielczuk H, Day M, DoNascimento V, Day M, Rodriguez I, van Essen-Zandbergen A, Schink AK, Wu G, Threlfall J, Woodward MJ, Coldham N, Kadlec K, Schwarz S, Dierikx C, Guerra B, Helmuth R, Mevius D, Woodford N, Wain J (2014a) Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerg Infect Dis 20(11):1935–1937

Chattaway MA, Jenkins C, Rajendram D, Cravioto A, Talukder KA, Dallman T, Underwood A, Platt S, Okeke IN, Wain J (2014b) Enteroaggregative *Escherichia coli* have evolved independently as distinct complexes within the *E. coli* population with varying ability to cause disease. PLoS One 9(11), e112967

Chaudhuri RR, Sebaihia M, Hobman JL, Webber MA, Leyton DL, Goldberg MD, Cunningham AF, Scott-Tucker A, Ferguson PR, Thomas CM, Frankel G, Tang CM, Dudley EG, Roberts IS, Rasko DA, Pallen MJ, Parkhill J, Nataro JP, Thomson NR, Henderson IR (2010) Complete genome sequence and comparative metabolic profiling of the prototypical enteroaggregative *Escherichia coli* strain 042. PLoS One 5(1), e8801

Cobeljic M, Miljkovic-Selimovic B, Paunovic-Todosijevic D, Velickovic Z, Lepsanovic Z, Zec N, Savic D, Ilic R, Konstantinovic S, Jovanovic B, Kostic V (1996) Enteroaggregative *Escherichia coli* associated with an outbreak of diarrhoea in a neonatal nursery ward. Epidemiol Infect 117(1):11–16

Cravioto A, Tello A, Navarro A, Ruiz J, Villafan H, Uribe F, Eslava C (1991) Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. Lancet 337(8736):262–264

Czeczulin JR, Balepur S, Hicks S, Phillips A, Hall R, Kothary MH, Navarro-Garcia F, Nataro JP (1997) Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. Infect Immun 65(10):4135–4145

Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F, Nataro JP (1999) Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. Infect Immun 67(6):2692–2699

Dallman TJ, Chattaway MA, Cowley LA, Doumith M, Tewolde R, Wooldridge DJ, Underwood A, Ready D, Wain J, Foster K, Grant KA, Jenkins C (2014) An investigation of the diversity of strains of enteroaggregative *Escherichia coli* isolated from cases associated with a large multi-pathogen foodborne outbreak in the UK. PLoS One 9(5), e98103

Dias RC, Dos Santos BC, Dos Santos LF, Vieira MA, Yamatogi RS, Mondelli AL, Sadatsune T, Sforcin JM, Gomes TA, Hernandes RT (2016) Diarrheagenic *Escherichia coli* pathotypes investigation revealed atypical enteropathogenic *E. coli* as putative emerging diarrheal agents in children living in Botucatu, Sao Paulo State, Brazil. APMIS 124(4):299–308

Dudley EG, Abe C, Ghigo JM, Latour-Lambert P, Hormazabal JC, Nataro JP (2006a) An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. Infect Immun 74(4):2102–2114

Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP (2006b) Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative *Escherichia coli*. Mol Microbiol 61(5):1267–1282

Elias WP, Suzart S, Trabulsi LR, Nataro JP, Gomes TA (1999) Distribution of aggA and aafA gene sequences among *Escherichia coli* isolates with genotypic or phenotypic characteristics, or both, of enteroaggregative *E. coli*. J Med Microbiol 48(6):597–599

Eslava C, Navarro-Garcia F, Czeczulin JR, Henderson IR, Cravioto A, Nataro JP (1998) Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. Infect Immun 66(7):3155–3163

Estrada-Garcia T, Navarro-Garcia F (2012) Enteroaggregative *Escherichia coli* pathotype: a genetically heterogeneous emerging foodborne enteropathogen. FEMS Immunol Med Microbiol 66(3):281–298

Farfan MJ, Inman KG, Nataro JP (2008) The major pilin subunit of the AAF/II fimbriae from enteroaggregative *Escherichia coli* mediates binding to extracellular matrix proteins. Infect Immun 76(10):4378–4384

Frank C, Werber D, Cramer JP, Askar M, Faber M, van der Heiden M, Bernard H, Fruth A, Prager R, Spode A, Wadl M, Zoufaly A, Jordan S, Kemper MJ, Follin P, Muller L, King LA, Rosner B, Buchholz U, Stark K, Krause G, Team HUSI (2011) Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. N Engl J Med 365(19):1771–1780

Friedrich AW, Bielaszewska M, Zhang WL, Pulz M, Kuczius T, Ammon A, Karch H (2002) *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis 185(1):74–84

Gohar A, Abdeltawab NF, Fahmy A, Amin MA (2016) Development of safe, effective and immunogenic vaccine candidate for diarrheagenic *Escherichia coli* main pathotypes in a mouse model. BMC Res Notes 9(1):80

Gomez-Duarte OG, Romero-Herazo YC, Paez-Canro CZ, Eslava-Schmalbach JH, Arzuza O (2013) Enterotoxigenic *Escherichia coli* associated with childhood diarrhoea in Colombia, South America. J Infect Dev Ctries 7(5):372–381

Gonzales L, Joffre E, Rivera R, Sjoling A, Svennerholm AM, Iniguez V (2013) Prevalence, seasonality and severity of disease caused by pathogenic *Escherichia coli* in children with diarrhoea in Bolivia. J Med Microbiol 62(Pt 11):1697–1706

Greenberg DE, Jiang ZD, Steffen R, Verenker MP, DuPont HL (2002) Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. J Infect Dis 185(7):944–949

Guerrant RL, Oria RB, Moore SR, Oria MO, Lima AA (2008) Malnutrition as an enteric infectious disease with long-term effects on child development. Nutr Rev 66(9):487–505

Gutierrez-Jimenez J, Arciniega I, Navarro-Garcia F (2008) The serine protease motif of Pic mediates a dose-dependent mucolytic activity after binding to sugar constituents of the mucin substrate. Microb Pathog 45(2):115–123

Harrington SM, Strauman MC, Abe CM, Nataro JP (2005) Aggregative adherence fimbriae contribute to the inflammatory response of epithelial cells infected with enteroaggregative *Escherichia coli*. Cell Microbiol 7(11):1565–1578

Harrington SM, Dudley EG, Nataro JP (2006) Pathogenesis of enteroaggregative *Escherichia coli* infection. FEMS Microbiol Lett 254(1):12–18

Harrington SM, Sheikh J, Henderson IR, Ruiz-Perez F, Cohen PS, Nataro JP (2009) The Pic protease of enteroaggregative *Escherichia coli* promotes intestinal colonization and growth in the presence of mucin. Infect Immun 77(6):2465–2473

Hebbelstrup Jensen B, Olsen KE, Struve C, Krogfelt KA, Petersen AM (2014) Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev 27(3):614–630

Henderson IR, Czeczulin J, Eslava C, Noriega F, Nataro JP (1999a) Characterization of pic, a secreted protease of *Shigella flexneri* and enteroaggregative *Escherichia coli*. Infect Immun 67(11):5587–5596

Henderson IR, Hicks S, Navarro-Garcia F, Elias WP, Philips AD, Nataro JP (1999b) Involvement of the enteroaggregative *Escherichia coli* plasmid-encoded toxin in causing human intestinal damage. Infect Immun 67(10):5338–5344

Herzog K, Engeler Dusel J, Hugentobler M, Beutin L, Sagesser G, Stephan R, Hachler H, Nuesch-Inderbinen M (2014) Diarrheagenic enteroaggregative *Escherichia coli* causing urinary tract infection and bacteremia leading to sepsis. Infection 42(2):441–444

Hicks S, Candy DC, Phillips AD (1996) Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. Infect Immun 64(11):4751–4760

Huang DB, Nataro JP, DuPont HL, Kamat PP, Mhatre AD, Okhuysen PC, Chiang T (2006) Enteroaggregative *Escherichia coli* is a cause of acute diarrheal illness: a meta-analysis. Clin Infect Dis 43(5):556–563

Huang DB, Mohamed JA, Nataro JP, DuPont HL, Jiang ZD, Okhuysen PC (2007) Virulence characteristics and the molecular epidemiology of enteroaggregative *Escherichia coli* isolates from travellers to developing countries. J Med Microbiol 56 (Pt 10):1386–1392. doi:56/10/1386 [pii]10.1099/jmm.0.47161-0

Itoh Y, Nagano I, Kunishima M, Ezaki T (1997) Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. J Clin Microbiol 35(10):2546–2550

Izquierdo M, Alvestegui A, Nataro JP, Ruiz-Perez F, Farfan MJ (2014a) Participation of integrin alpha5beta1 in the fibronectin-mediated adherence of enteroaggregative *Escherichia coli* to intestinal cells. Biomed Res Int 2014:781246

Izquierdo M, Navarro-Garcia F, Nava-Acosta R, Nataro JP, Ruiz-Perez F, Farfan MJ (2014b) Identification of cell surface-exposed proteins involved in the fimbria-mediated adherence of enteroaggregative *Escherichia coli* to intestinal cells. Infect Immun 82(4):1719–1724

Jenkins C, Chart H, Willshaw GA, Cheasty T, Smith HR (2006) Genotyping of enteroaggregative *Escherichia coli* and identification of target genes for the detection of both typical and atypical strains. Diagn Microbiol Infect Dis 55(1):13–19

Jiang ZD, Greenberg D, Nataro JP, Steffen R, DuPont HL (2002) Rate of occurrence and pathogenic effect of enteroaggregative *Escherichia coli* virulence factors in international travelers. J Clin Microbiol 40(11):4185–4190

Jiang ZD, Okhuysen PC, Guo DC, He R, King TM, DuPont HL, Milewicz DM (2003) Genetic susceptibility to enteroaggregative *Escherichia coli* diarrhea: polymorphism in the interleukin-8 promotor region. J Infect Dis 188(4):506–511

Jimenez Gutierrez E, Pineda V, Calzada JE, Guerrant RL, Lima Neto JB, Pinkerton RC, Saldana A (2014) Enteric parasites and enteroaggregative *Escherichia coli* in children from Canazas County, Veraguas Province, Panama. Am J Trop Med Hyg 91(2):267–272

Jonsson R, Struve C, Boisen N, Mateiu RV, Santiago AE, Jenssen H, Nataro JP, Krogfelt KA (2015) Novel aggregative adherence fimbria variant of enteroaggregative *Escherichia coli*. Infect Immun 83(4):1396–1405

Jose J, Meyer TF (2007) The autodisplay story, from discovery to biotechnical and biomedical applications. Microbiol Mol Biol Rev 71(4):600–619

Mancini J, Weckselblatt B, Chung YK, Durante JC, Andelman S, Glaubman J, Dorff JD, Bhargava S, Lijek RS, Unger KP, Okeke IN (2011) The heat-resistant agglutinin family includes a novel adhesin from enteroaggregative *Escherichia coli* strain 60A. J Bacteriol 193(18):4813–4820

Mathewson JJ, Johnson PC, DuPont HL, Satterwhite TK, Winsor DK (1986) Pathogenicity of enteroadherent *Escherichia coli* in adult volunteers. J Infect Dis 154(3):524–527

Medina AM, Rivera FP, Romero LM, Kolevic LA, Castillo ME, Verne E, Hernandez R, Mayor YE, Barletta F, Mercado E, Ochoa TJ (2010) Diarrheagenic *Escherichia coli* in human immunodeficiency virus (HIV) pediatric patients in Lima, Peru. Am J Trop Med Hyg 83(1):158–163

Mohamed JA, DuPont HL, Flores J, Palur H, Nair P, Jiang ZD, Guo D, Belkind-Gerson J, Okhuysen PC (2011) Single nucleotide polymorphisms in the promoter of the gene encoding the lipopolysaccharide receptor CD14 are associated with bacterial diarrhea in US and Canadian travelers to Mexico. Clin Infect Dis 52(11):1332–1341

Morin N, Santiago AE, Ernst RK, Guillot SJ, Nataro JP (2013) Characterization of the AggR regulon in enteroaggregative *Escherichia coli*. Infect Immun 81(1):122–132

Munera D, Ritchie JM, Hatzios SK, Bronson R, Fang G, Schadt EE, Davis BM, Waldor MK (2014) Autotransporters but not pAA are critical for rabbit colonization by Shiga toxin-producing *Escherichia coli* O104:H4. Nat Commun 5:3080

Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P, Levine MM (1987) Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr Infect Dis J 6(9):829–831

Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM (1992) Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun 60(6):2297–2304

Nataro JP, Deng Y, Cookson S, Cravioto A, Savarino SJ, Guers LD, Levine MM, Tacket CO (1995) Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. J Infect Dis 171(2):465–468

Nataro JP, Hicks S, Phillips AD, Vial PA, Sears CL (1996) T84 cells in culture as a model for enteroaggregative *Escherichia coli* pathogenesis. Infect Immun 64(11):4761–4768

Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, Edberg SC, Braden CR, Glenn Morris J Jr, Hirshon JM (2006) Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven, Connecticut. Clin Infect Dis 43(4):402–407

Nava-Acosta R, Navarro-Garcia F (2013) Cytokeratin 8 is an epithelial cell receptor for Pet, a cytotoxic serine protease autotransporter of *Enterobacteriaceae*. mBio 4(6):e00838–e008313

Navarro-Garcia F (2014) *Escherichia coli* O104:H4 pathogenesis: an enteroaggregative *E. coli*/*Shiga* toxin-producing *E. coli* explosive cocktail of high virulence. Microbiol Spectr 2(6)

Navarro-Garcia F, Elias WP (2011) Autotransporters and virulence of enteroaggregative *E. coli*. Gut Microbes 2(1):13–24

Navarro-Garcia F, Eslava C, Villaseca JM, Lopez-Revilla R, Czeczulin JR, Srinivas S, Nataro JP, Cravioto A (1998) *In vitro* effects of a high-molecular-weight heat-labile enterotoxin from enteroaggregative *Escherichia coli*. Infect Immun 66(7):3149–3154

Navarro-Garcia F, Sears C, Eslava C, Cravioto A, Nataro JP (1999) Cytoskeletal effects induced by pet, the serine protease enterotoxin of enteroaggregative *Escherichia coli*. Infect Immun 67(5):2184–2192

Navarro-Garcia F, Gutierrez-Jimenez J, Garcia-Tovar C, Castro LA, Salazar-Gonzalez H, Cordova V (2010) Pic, an autotransporter protein secreted by different pathogens in the *Enterobacteriaceae* family, is a potent mucus secretagogue. Infect Immun 78(10):4101–4109

Nishi J, Sheikh J, Mizuguchi K, Luisi B, Burland V, Boutin A, Rose DJ, Blattner FR, Nataro JP (2003) The export of coat protein from enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system. J Biol Chem 278(46):45680–45689

Olesen B, Scheutz F, Andersen RL, Menard M, Boisen N, Johnston B, Hansen DS, Krogfelt KA, Nataro JP, Johnson JR (2012) Enteroaggregative *Escherichia coli* O78:H10, the cause of an outbreak of urinary tract infection. J Clin Microbiol 50(11):3703–3711

Oloomi M, Bouzari S, Emami S (2009) A recombinant hybrid peptide composed of AAF adhesin of enteroaggregative *Escherichia coli* and *Shiga* toxin B subunit elicits protective immune response in mice. Eur J Clin Microbiol Infect Dis 28(11):1311–1316

Pabalan N, Singian E, Jarjanazi H, Steiner TS (2013) Enteroaggregative *Escherichia coli* and acute diarrhea in children: a meta-analysis of South Asian populations. Eur J Clin Microbiol Infect Dis 32(5):597–607

Panchalingam S, Antonio M, Hossain A, Mandomando I, Ochieng B, Oundo J, Ramamurthy T, Tamboura B, Zaidi AK, Petri W, Houpt E, Murray P, Prado V, Vidal R, Steele D, Strockbine N, Sansonetti P, Glass RI, Robins-Browne RM, Tauschek M, Svennerholm AM, Berkeley LY, Kotloff K, Levine MM, Nataro JP (2012) Diagnostic microbiologic methods in the GEMS-1 case/control study. Clin Infect Dis 55(suppl 4):S294–S302

Patzi-Vargas S, Zaidi MB, Perez-Martinez I, Leon-Cen M, Michel-Ayala A, Chaussabel D, Estrada-Garcia T (2015) Diarrheagenic *Escherichia coli* carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis 9(3), e0003510

Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJ, McGrath M, Olortegui MP, Samie A, Shakoor S, Mondal D, Lima IF, Hariraju D, Rayamajhi BB, Qureshi S,

Kabir F, Yori PP, Mufamadi B, Amour C, Carreon JD, Richard SA, Lang D, Bessong P, Mduma E, Ahmed T, Lima AA, Mason CJ, Zaidi AK, Bhutta ZA, Kosek M, Guerrant RL, Gottlieb M, Miller M, Kang G, Houpt ER, Investigators M-EN (2015) Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). Lancet Glob Health 3(9):e564–e575

Polotsky Y, Nataro JP, Kotler D, Barrett TJ, Orenstein JM (1997) HEp-2 cell adherence patterns, serotyping, and DNA analysis of *Escherichia coli* isolates from eight patients with AIDS and chronic diarrhea. J Clin Microbiol 35(8):1952–1958

Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, Paxinos EE, Sebra R, Chin CS, Iliopoulos D, Klammer A, Peluso P, Lee L, Kislyuk AO, Bullard J, Kasarskis A, Wang S, Eid J, Rank D, Redman JC, Steyert SR, Frimodt-Moller J, Struve C, Petersen AM, Krogfelt KA, Nataro JP, Schadt EE, Waldor MK (2011) Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. N Engl J Med 365(8):709–717

Roche JK, Cabel A, Sevilleja J, Nataro J, Guerrant RL (2010) Enteroaggregative *Escherichia coli* (EAEC) impairs growth while malnutrition worsens EAEC infection: a novel murine model of the infection malnutrition cycle. J Infect Dis 202(4):506–514

Ross BN, Rojas-Lopez M, Cieza RJ, McWilliams BD, Torres AG (2015) The role of long polar fimbriae in *Escherichia coli* O104:H4 adhesion and colonization. PLoS One 10(10), e0141845

Ruiz-Perez F, Nataro JP (2014) Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence. Cell Mol Life Sci 71(5):745–770

Ruiz-Perez F, Wahid R, Faherty CS, Kolappaswamy K, Rodriguez L, Santiago A, Murphy E, Cross A, Sztein MB, Nataro JP (2011) Serine protease autotransporters from *Shigella flexneri* and pathogenic *Escherichia coli* target a broad range of leukocyte glycoproteins. Proc Natl Acad Sci U S A 108(31):12881–12886

Savarino SJ, Fasano A, Robertson DC, Levine MM (1991) Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. J Clin Invest 87(4):1450–1455

Scavia G, Staffolani M, Fisichella S, Striano G, Colletta S, Ferri G, Escher M, Minelli F, Caprioli A (2008) Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. J Med Microbiol 57(Pt 9):1141–1146

Scholl D, Gebhart D, Williams SR, Bates A, Mandrell R (2012) Genome sequence of *E. coli* O104:H4 leads to rapid development of a targeted antimicrobial agent against this emerging pathogen. PLoS One 7(3), e33637

Sheikh J, Czeczulin JR, Harrington S, Hicks S, Henderson IR, Le Bouguenec C, Gounon P, Phillips A, Nataro JP (2002) A novel dispersin protein in enteroaggregative *Escherichia coli*. J Clin Invest 110(9):1329–1337

Sheikh J, Dudley EG, Sui B, Tamboura B, Suleman A, Nataro JP (2006) EilA, a HilA-like regulator in enteroaggregative *Escherichia coli*. Mol Microbiol 61(2):338–350

Steiner TS, Lima AA, Nataro JP, Guerrant RL (1998) Enteroaggregative *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. J Infect Dis 177(1):88–96

Steiner TS, Nataro JP, Poteet-Smith CE, Smith JA, Guerrant RL (2000) Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J Clin Invest 105(12):1769–1777

Tapia-Pastrana G, Chavez-Duenas L, Lanz-Mendoza H, Teter K, Navarro-Garcia F (2012) VirK is a periplasmic protein required for efficient secretion of plasmid-encoded toxin from enteroaggregative *Escherichia coli*. Infect Immun 80(7):2276–2285

Varela G, Batthyány L, Bianco MN, Pérez W, Pardo L, Algorta G, Robino L, Suárez R, Navarro A, Pírez MC, Schelotto F (2015) Enteropathogens associated with acute diarrhea in children from households with high socioeconomic level in Uruguay. Int J Microbiol 2015:592953

Vial PA, Robins-Browne R, Lior H, Prado V, Kaper JB, Nataro JP, Maneval D, Elsayed A, Levine MM (1988) Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. J Infect Dis 158(1):70–79

Chapter 3
Typical Enteropathogenic *Escherichia coli*

Isabel C.A. Scaletsky and Ulysses Fagundes-Neto

Summary Enteropathogenic Escherichia coli (EPEC) is a leading cause of infantile diarrhea in developing countries. EPEC colonizes the small intestine and causes attaching-and-effacing (A/E) lesions, a phenotype encoded on the locus of enterocyte effacement (LEE) pathogenicity island. LEE encodes the components of a type III secretion system, various effector molecules, and the intimin gene (eae). Typical EPEC strains contain, in addition to eae, the EPEC adherence factor (EAF) plasmid, which encodes the bundle-forming pili that mediate localized adherence to epithelial cells, whereas atypical do not possess this plasmid. The exact mechanism of diarrhea production is not fully understood. Diagnosis of EPEC is now based on molecular methods to detect virulence characteristics. EPEC-induced diarrhea is self-limiting, and oral rehydration is effective.

I.C.A. Scaletsky (✉)
Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Rua Botucatu, 862, 3 andar, Vila Clementino, 04023-062 São Paulo, São Paulo, Brazil
E-mail: scaletsky@unifesp.br

U. Fagundes-Neto
Departamento de Pediatria, Universidade Federal de São Paulo,
Rua Botucatu, 598 Vila Clementino, 04023-062 São Paulo, São Paulo, Brazil
E-mail: ulyneto@osite.com.br

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_3

1 Definition of EPEC

The term EPEC was first used in 1955 by Neter et al. (1955) to describe a number of *E. coli* strains epidemiologically related to a series of outbreaks of infantile diarrhea in the 1940s and 1950s (Bray 1945; Robins-Browne 1987). Originally defined by serotype, EPEC are now defined as those having the ability to cause diarrhea, the ability to produce a histopathology on the intestinal epithelium known as the attaching and effacing (A/E) lesion, and the inability to produce Shiga toxins (Nataro and Kaper 1998). Typical EPEC strains of human origin causing infectious diarrhea possess a large virulence plasmid known as the EPEC adherence factor (EAF) plasmid, which encodes localized adherence on cultured epithelial cells mediated by the bundle-forming pilus (BFP), while atypical EPEC do not possess this plasmid (Nataro and Kaper 1998).

2 Clonal Lineages of EPEC

The majority of typical EPEC strains fall into well-recognized O serotypes. Classic EPEC O serogroups include O55, O86, O111, O114, O119, O127, and O142. The most common H antigens associated with EPEC are the H6 and the H2 antigens. A less common EPEC type is H34, and a number of typical EPEC strains are nonmotile in conventional tests and classified as H–. Typical EPEC strains belonging to nonclassic serotypes have also been reported (Trabulsi et al. 2002). Based on multilocus enzyme electrophoresis analysis of allelic differences among housekeeping genes, typical EPEC strains have been subtyped into two major lineages, previously designated EPEC1 and EPEC2 (Whittam and McGraw 1996). The EPEC1 includes widespread serotypes such as O55:H6 and O119:H6, whereas EPEC2 consists of serotypes with more limited occurrence such as O111:H2 and O114:H2. Based on a whole-genome phylogeny and analysis of type III secretion system effectors, typical EPEC strains have been demonstrated to cluster in three main phylogenomic lineages, designated EPEC1, EPEC2, and EPEC4 (Hazen et al. 2013).

3 Epidemiology of EPEC Infection

3.1 Incidence

EPEC strains were first identified as the cause of outbreaks associated with infantile diarrhea in the United States and the United Kingdom (Bray 1945; Neter et al. 1955; Robins-Browne 1987). After that period, EPEC strains were rarely detected in these countries, but they became a major cause of infantile diarrhea in numerous developing countries (Nataro and Kaper 1998). The prevalence of EPEC infection varies between epidemiological studies based on differences in study populations, age, distributions, and methods used for detection and diagnosis (Ochoa et al. 2008). In

addition, geographic region and socioeconomic class may also contribute to the epidemiology of EPEC-induced diarrheal disease (Maranhão et al. 2008). The occurrence of diarrhea due to typical EPEC decreases with age, and infections in adults are rarely reported. This apparent resistance in adults and older children has been attributed to the loss of specific receptors with age or development of immunity (Nataro and Kaper 1998).

For many decades, studies conducted worldwide have shown that typical EPEC serotypes were strongly associated with diarrhea in children <1 year of age, mainly in poor children of urban centers (Nataro and Kaper 1998). The association with diarrhea was particularly strong in infants less than 6 months of age. Studies in Brazil, Chile, Mexico, and South Africa have shown that 30–40 % of infantile diarrhea were caused by typical EPEC serotypes (Ochoa et al. 2008). However, the epidemiology of EPEC infection has shifted. In numerous developing countries, where the prevalence of EPEC infection had been high until the 1990s, recent studies have not identified a significant association between typical EPEC and infantile diarrhea. In the meantime, the proportion of atypical EPEC strains has increased and outnumbered typical EPEC strains, and atypical EPEC strains have also been associated with childhood diarrhea in both developing and developed countries (Ochoa et al. 2008; Hu and Torres 2015). In Brazil, 92 % of EPEC isolates collected from children between 2001 and 2002 were atypical (Franzolin et al. 2005), compared to 38 % in a 1998–1999 study (Scaletsky et al. 2010). A 5-year study of children under 12 years of age in Thailand found that 71.8 % of EPEC isolates were atypical strains (Ratchtrachenchai et al. 2004). Likewise, 39.9 % of EPEC strains isolated from children with diarrhea in Iran were typical, while the remaining 61.7 % were atypical (Bakhshi et al. 2013). Studies from Norway (Afset et al. 2003) and Australia (Nguyen et al. 2006) also suggest that atypical isolates are more commonly found among persistent cases of diarrhea than typical isolates. However, other studies still report typical being more prevalent than atypical EPEC as a cause of diarrhea (Alikhani et al. 2006). Recent estimates from the Centers for Disease Control and Prevention (CDC) on food-related illness in the United States listed only four hospitalizations as a result of EPEC infection (CDC 2013).

Overall, the importance of EPEC as a cause of diarrhea has declined in published literature of the last several decades (Ochoa et al. 2008). The reasons for such decrease probably comprise better control of hospital infections, improvements in public health measures such as active interventions, therapy sanitary conditions, and control of hospital infections (Trabulsi et al. 2002). Based on a systematic review of pediatric diarrhea etiology using 266 studies published between 1990 and 2002, EPEC are still among the most important pathogens (Ochoa and Contreras 2011). Likewise, in 2014 a European multicenter prospective quarterly point-prevalence study of community-acquired diarrhea (EUCODI) showed that EPEC is highly prevalent during both the first with a median prevalence of 8.8 % in the community setting, 9.1 % in the outpatient setting, and 15.6 % in the impatient setting (Spina et al. 2015). However, there are important regional and temporal variations. In a recent study of hospitalized patients with diarrhea in India, EPEC was responsible for 3.2 % of 648 diarrhea samples in children younger than 5 years of age (Nair et al. 2010).

3.2 Reemergence

The occurrence of severe disease outcomes associated with typical EPEC has reemerged. The recently completed Global Enteric Multicenter Study (GEMS) was a prospective, population-based case-control study involving seven sites in Africa and Asia with the goal of identifying the etiology, burden, and associated mortality related to acute moderate-to-severe diarrhea in children less than 5 years of age (Kotloff et al. 2013). Based on GEMS data, typical EPEC was significantly associated with moderate to severe diarrhea in children under 2 years of age in Kenya, whereas atypical EPEC was not associated with this type of diarrhea (Kotloff et al. 2013). Overall, typical EPEC was not strongly associated with cases of moderate to severe diarrhea, but when present, it was associated with an increased risk of death in infants (Kotloff et al. 2013).

3.3 Transmission and Reservoirs

Transmission of EPEC follows a fecal-oral process through contaminated surfaces, weaning fluids, and human carriers (Levine and Edelman 1984). Although rare, outbreaks among adults seem to occur through ingestion of contaminated food and water; however, no specific environmental reservoir has been identified (Nataro and Kaper 1998). The infective dose in adult volunteers is high, at 10^8–10^{10} organisms (Levine et al. 1978), while the infective dose to cause disease in children is unknown. EPEC outbreaks have been reported to show a seasonal distribution with peaks during the warm months (Nataro and Kaper 1998; Behiry et al. 2011). Humans are the only known reservoir for typical EPEC, with symptomatic and asymptomatic children and asymptomatic adults being the most likely source (Nataro and Kaper 1998).

4 Phenotypes Characteristic of EPEC

4.1 Localized Adherence to Epithelial Cells

Typical EPEC strains adhere to HeLa, HEp-2, and other cell lines and to organ cultures in vitro in a distinctive three-dimensional microcolonies pattern, the so-called localized adherence (LA) phenotype (Fig. 3.1a) (Scaletsky et al. 1984; Nataro and Kaper 1998). A similar adherence pattern has been seen in tissue biopsies of EPEC-infected humans (Rothbaum et al. 1982). The LA phenotype is mediated by the plasmid-borne type IV fimbriae commonly known as bundle-forming pilus (BFP) (Girón et al. 1991). EPEC adherence is inhibited by various sugar moieties, including galactose, *N*-acetylgalactosamine, *N*-acetyllactosamine, and fucosylated oligosaccharides and gangliosides from milk. These saccharides could be moieties of host

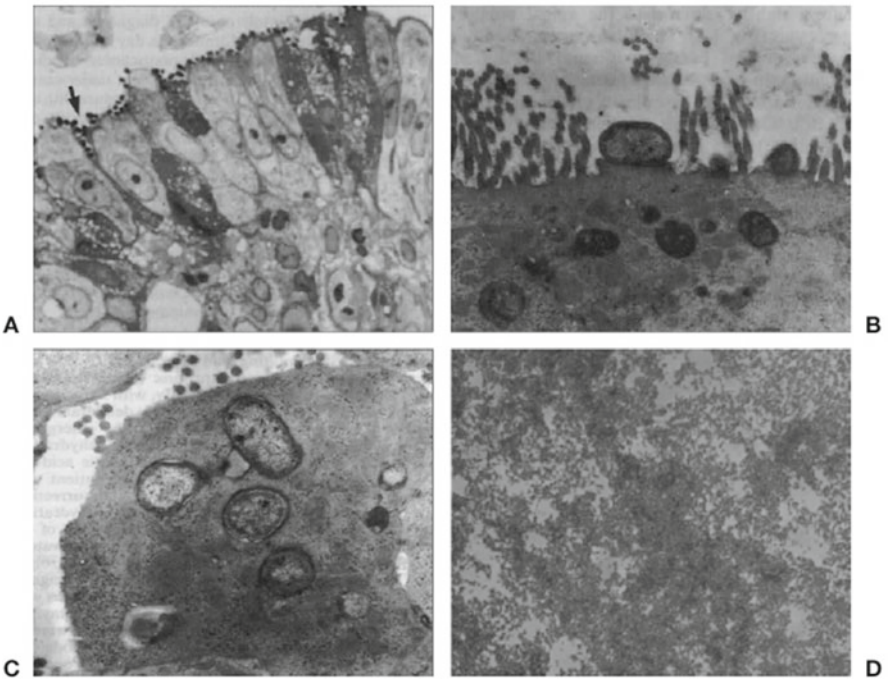

Fig. 3.1 Photomicrography and electron-micrograph of the small bowel biopsy of infant infected with typical EPEC O111ab:H2 (Fagundes-Neto et al. 1995). (**a**) Adherent bacteria seen in the small bowel biopsy of patient (*arrow*). (**b**) Effacing of the microvilli and membrane cupping, with pedestal formation at the sites of bacterial attachment. (**c**) Bacteria are seen in the interior of the enterocyte. (**d**) Light microscopy of biofilm formation by typical EPEC O111 on an abiotic surface (Photo courtesy of Heloisa H. Nascimento)

cell glycoproteins that serve as EPEC receptors. EPEC also bind to phosphatidyl-ethanolamine, as a component of cell membrane (Nougayrède et al. 2003).

4.2 Attaching and Effacing Lesion

A hallmark phenotype of EPEC is the ability to produce attaching and effacing (A/E) lesions (Moon et al. 1983). This phenotype is characterized by effacement of intestinal epithelial-cell microvilli and intimate adherence between the bacterium and the epithelial cell membrane (Fig. 3.1b). Directly beneath the adherent bacterium, marked cytoskeletal changes are seen in the epithelial cell membrane, particularly the formation of an actin-rich, cup-like pedestal, at the site of bacterial contact. The A/E lesions are observed in model EPEC infections with cultured cells and mucosal explants as well as in intestinal biopsies from EPEC-infected infants or animals (Nataro and Kaper 1998).

4.3 Invasion of Epithelial Cells

Intracellular EPEC have been observed both in tissue culture and in small intestinal biopsies from an EPEC-infected infant (Nataro and Kaper 1998) (Fig. 3.1c). Two studies have reported that O111:NM strains contain plasmid sequences that confer invasiveness upon *E. coli* K12 strains containing the clone fragments (Fletcher et al. 1992; Scaletsky et al. 1995). Sequences homologous to these cloned genes were present in only a minority of EPEC strains. Despite their invasive potential, EPEC are considered to be extracellular pathogens.

4.4 Autoaggregation/Biofilm Formation

Typical EPEC have the ability to form tight, spherical, bacterial auto-aggregates when cultured in liquid culture (Nougayrède et al. 2003). Like LA, autoaggregation requires BFP (see Sect. 5.2). Typical EPEC also form biofilms on abiotic surfaces under static conditions (Fig. 3.1d), or on a flow through continuous culture system, and a model of EPEC biofilm formation has been proposed (Moreira et al. 2006). Transposon mutagenesis analysis identified adhesive structures such as type 1 pili, antigen 43, BFP, and the EspA filament (see Sects. 5.2 and 5.3) as participants in bacterial aggregation during biofilm formation on abiotic surfaces (Moreira et al. 2006).

5 EPEC Virulence Factors and Genomics

5.1 The EAF Plasmid

Typical EPEC strains harbor a 50–70 MDa virulence plasmid known as the EPEC adherence factor (EAF) plasmid (Baldini et al. 1983), which varies in sequence among different EPEC strains but is somewhat conserved (Nataro et al. 1987). Curing of the EAF plasmid from EPEC abolishes the LA and the autoaggregation phenotypes, and leads to attenuated virulence in volunteers (Nataro and Kaper 1998). The self-transmissible EAF plasmid pMAR2 is found among strains of the EPEC1 lineage and contains an intact transfer region, unlike pB171, which is more common among EPEC2 strains (Tobe et al. 1999; Brinkley et al. 2006). Two loci important for pathogenicity have been located on the EAF plasmid: the *bfp* gene cluster encoding BFP (Girón et al. 1991) and the *per* locus, encoding a transcriptional activator called plasmid-encoded regulator (Per) (Tobe et al. 1999). Between pMAR2 and pB171, the *bfp* and *per* loci share 99 % sequence similarity (Brinkley et al. 2006), and both BFP and PerA have been shown to contribute to virulence in human volunteers (Bieber et al. 1998).

Comparative genomics of the EAF plasmids from diverse EPEC phylogenomic lineages demonstrated significant plasmid diversity even among isolates within the

same phylogenomic lineage (Hazen et al. 2015). In some instance, EPEC isolates have likely lost their ancestral EAF plasmid and have acquired another EAF plasmid originating from a different lineage of *E. coli* Also, the EAF plasmids have undergone genetic modifications, including recombination of the *bfp* genes and other genes on the plasmid (Hazen et al. 2015). In addition, global transcriptional analyses of the EPEC1 prototype isolate E2348/69 and its EAF plasmid-free (JPN15) and Δ*perABC* mutants demonstrated that plasmid genes other than *perABC* influence the expression of a number of both plasmid and chromosomal genes under virulence-inducing conditions (Hazen et al. 2015).

5.2 Bundle-Forming Pilus

Typical EPEC strains produce a type IV pilus, the bundle-forming pili (BFP) which is responsible for the LA phenotype, and also contributes to antigenicity, autoaggregation, and biofilm formation (Girón et al. 1991; Bieber et al. 1998; Vuopio-Varkila and Schoolnik 1991; Moreira et al. 2006; Hyland et al. 2008). An operon of 14 genes contained on the EAF plasmid is necessary for expression of the BFP, with *bfpA* encoding the major structural subunit (bundlin) (Stone et al. 1996). The *bfp* genes are highly conserved among EPEC1 and EPEC2 strains, but some O128:H2 and O119:H2 EPEC strains that contain part of the *bfpA* gene have the rest of the *bfp* genes cluster deleted. Adjacent to the deleted region is an IS*66* element, which is proposed to play a role in the deletion of the *bfp* operon in these strains (Bortoloni et al. 1999; Teixeira et al. 2015).

5.3 The LEE Pathogenicity Island, the Type III Secretion System, and Translocated Proteins

The locus of enterocyte effacement (LEE) is a 35-kb pathogenicity island of EPEC containing genes required for the formation of A/E lesion (McDaniel et al. 1995). The EPEC LEE is organized into five operons (*LEE1* to *LEE5*) (Elliott et al. 2000; Deng et al. 2004; Dean and Kenny 2009; Croxen et al. 2013). The *LEE1*, *LEE2*, and *LEE3* operons encode components of a type III protein secretion system (T3SS), and the global regulators Ler (LEE-encoded regulator), GrlA (global regulator of LEE activator; formerly called Orf11), and GrlR (Grl repressor; formerly called Orf10) (Barba et al. 2005). *LEE4* encodes T3SS-secreted proteins EspA, EspB, and EspD (EPEC-secreted protein) that are also components of the translocation apparatus by which other effector proteins are translocated into the cell. *LEE5* encodes the adhesin intimin and its translocated receptor the Tir (see Sect. 5.4) (Kenny et al. 1997).

In addition to Tir, the EPEC genome contains other six LEE-encoded effector proteins translocated into the cell (Map, EspF, EspG, EspZ, EspH, and EspB), and they interfere with different aspects of the cell physiology (Deng et al. 2004; Dean

and Kenny 2009; Croxen et al. 2013). Map, mitochondrial-associated protein, stimulates formation of membrane filopodia and epithelial barrier disruption as well as mitochondrial dysfunction. Multifunctional properties have also been reported for EspF and EspG, both of which affect aquaporin localization, leading to diarrhea. Like Map, EspF localizes to mitochondria and has been shown to disrupt tight junctions, while EspG alters host cytoskeletal components through its interaction with tubulin. The EspZ protein promotes host cell survival, whereas EspH affects filopodium formation, participates in actin signaling during pedestal formation and acts as a RhoGEF inhibitor. Both EspH and EspB are capable of inhibitingphagocytosis of EPEC by macrophages (Santos and Finlay 2015).

In addition to the LEE effectors, the prototype EPEC strain E2348/69 encodes at least 23 non-LEE (Nle)-encoded effector genes (Deng et al. 2004; Santos and Finlay 2015), many of which are involved in dampening the host immune response. NleB, NleC, NleD, NleE, and NleH have all been shown to inhibit NF-Kβ activation through a variety of different mechanisms. In addition to immunomodulatory functions, Nle effectors such as EspJ have anti-phagocytic properties, while NleA alters host protein secretion and tight junction integrity and inhibits vesicle trafficking. NleH is capable of modulating apoptotic responses (Santos and Finlay 2015).

5.4 Intimin and Tir

Intimin is a 94-kDa protein encoded by the *eae* gene and required for intimate adherence of EPEC to host cells at the sites of A/E lesions (Nataro and Kaper 1998). The N-terminus of intimin is highly conserved, whereas the C-terminus is highly variable (Frankel et al. 1994). Differences in the C-terminus of intimin have been used as a basis for classification into several distinct subtypes [represented by the Greek letters to α through ζ (zeta)] (Lacher et al. 2006, 2007). The α subtype is expressed by EPEC1 strains while subtype β is associated with human EPEC2 strains. The N-terminus of intimin anchors the protein in the EPEC outer membrane, whereas the C-terminus extends from the EPEC surface, binds to the translocated intimin receptor, Tir (Kenny et al. 1997). Intimin-Tir interaction leads to intimate adherence and pedestal formation beneath adherent bacteria (Kenny et al. 1997). Tir has also been shown to inhibit NF-κB activity through tumor necrosis factor alpha (TNF-α) receptor-associated factors (Ruchaud-Sparagano et al. 2011).

5.5 Lymphostatin

Typical EPEC strains encode a large surface protein, lymphocyte inhibitory factor (LifA), which inhibits the expression of multiple lymphokines and inhibits lymphocyte proliferation (Klapproth et al. 2000). Related genes *efa1* (a nearly identical

chromosomal locus from an enterohemorrhagic *E. coli* [EHEC] serogroup O111 strain) and *toxB* (a locus with significant homology found on the EHEC serotype O157:H7 plasmid) were implicated in adhesion to epithelial cells (Nicholls et al. 2000). There is evidence indicating that Efa1/LifA contributes to epithelial cell adherence in vitro (Badea et al. 2003) and is required for intestinal colonization of mice by the related A/E pathogen *Citrobacter rodentium* (Klapproth et al. 2004).

5.6 EspC

EspC is a large (110 kDa) protein that is encoded in a 15-kb chromosomal island specific to EPEC1 strains, and unlike proteins secreted by the T3SS, EspC secretion is mediated by the type V secretion system (Nataro and Kaper 1998; Vidal and Navarro-Garcia 2008). EspC acts as an enterotoxin, causing cytopathic effects on epithelial cells, including cytoskeletal damage (Navarro-Garcia et al. 2004). EspC cytotoxicity depends on its internalization and functional serine protease motif (Vidal and Navarro-Garcia 2008). EspC has been shown to enter intestinal epithelial cells through a cooperative mechanism involving both the T5SS and the T3SS (Vidal and Navarro-Garcia 2008). EspC confers enhanced lysozyme resistance to EPEC and purified EspC has been shown to interact with and degrade hemoglobin (Drago-Serrano et al. 2006) and to hydrolyze other proteins such as pepsin, factor V, and spectrin (Salinger et al. 2009). In addition, oligomerization of EspC gives rise to rope-like structures that serve as a substratum for adherence and biofilm formation as well as to protect bacteria form antimicrobial compounds (Xicohtencatl-Cortes et al. 2010).

5.7 Other Toxins

Some EPEC strains harbor the *astA* gene which encodes for the enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin 1 (EAST1) (Yamamoto et al. 1997; Dulguer et al. 2003). A recent study reported that 11 of 70 (16%) typical EPEC strains tested harbored an intact *astA* gene (Silva et al. 2014). EPEC strains of serotype O86:H34 produce cytolethal distending toxin (CDT) (Nataro and Kaper 1998). The mechanism of action of this toxin involves chromatin disruption, which leads to G2/M-phase growth arrest of the target cell and ultimately cell death (Lara-Tejero and Galan 2000). The significance of EAST1 and CDT toxins in EPEC pathogenesis remains unknown.

5.8 Other Fimbriae

Some EPEC strains possess other fimbriae or pili in addition to BFP. Type 1 fimbriae of EPEC have been found to be antigenic in volunteer studies; however, do not have a role in adherence to epithelial cells in vitro (Nataro and Kaper 1998). Two other

EPEC surface structures, rod-like fimbriae and fibrillae, have been characterized and have been suggested to be involved in the interaction of EPEC with host cells (Girón et al. 1993), but the role of these fimbriae in EPEC pathogenesis, has yet to be understood. In addition, some EPEC strains have conserved fimbrial genes encoding homologs of the long polar fimbriae (LPF) (Tatsuno et al. 2006). The *lpf* region (*lpfABCDE*) in typical EPEC encodes predicted proteins with about 60 % homology to the LPF of *Salmonella*, but initial studies have indicated that LPF is apparently not necessary for adherence and A/E lesion on human biopsies (Tatsuno et al. 2006). A number of polymorphisms within the *lpfA* genes have been recently identified among EPEC strains (Torres et al. 2004). The *E. coli* common pilus (ECP) has also been shown to act as an accessory adherence factor in EPEC, playing a role during cell adherence and/or in bacterium–bacterium interactions (Saldaña et al. 2009). However, the significance of ECP to EPEC pathogenesis has not been determined.

5.9 Flagella

A study has suggested that flagella may also be involved in EPEC adherence to epithelial cells (Girón et al. 2002). EPEC mutants with mutations in the flagellar gene *fliC* were markedly impaired in their ability to adhere and form microcolonies. Furthermore, purified EPEC flagella and anti-flagellum antibodies were both effective in blocking the adherence of several EPEC serotypes (Girón et al. 2002). However, another study could not confirm a role of flagella in EPEC adherence (Clearly et al. 2004).

6 Regulation of Virulence Factors

The expression of EPEC virulence factors is regulated by Per (plasmid-encoded regulator) which activates the transcription of several genes in the chromosome and on the EAF plasmid. Per activates *ler* (for LEE-encoded regulator), the first gene in the *LEE1* operon, which in turn activates the additional LEE operons (*LEE2*, *LEE3*, *LEE4*, and *LEE5*), and the LEE genes *espF*, *espG*, and *map* (Elliott et al. 2000; Deng et al. 2004; Dean and Kenny 2009). The Ler protein is a distant homolog of H-NS (for histone-like nucleoid-structuring protein), a nucleoid-associated protein that is frequently involved in the response of enterobacteria to environmental stimuli. Additionally, Ler controls genes located outside the LEE, such as *espC* and *nleA* (Elliott et al. 2000). It has also been shown that quorum sensing controls expression of the EPEC LEE (Sperandio et al. 2002). Regulation of *LEE1* by quorum sensing in turn increases the expression of the *LEE3* and *LEE4* operons via Ler. Additional regulators include the integration host factor (IHF), Bip, a tyrosine-phosphorylated GTPase, the Fis (factor for inversion stimulation), and GadX, which is a member of the AraC transcription factor family (Dean and Kenny 2009). Two novel LEE-encoded regulators have been identified, GrlA (global regulator of LEE activator)

and GrlR (Grl repressor), that have roles in *ler* expression (Barba et al. 2005). GrlR and GrlA are positive and negative regulators, respectively, required for the expression of several LEE-encoded genes (Barba et al. 2005).

7 EPEC Pathogenesis

The early and later events in EPEC adhesion and pathogenesis have been proposed to occur in distinct stages, including initial adherence to the host cell, signal transduction, and intimate attachment with pedestal formation (Fig. 3.2) (DeVinney et al. 1999; Donnenberg and Kaper 1992; Nougayrède et al. 2003; Torres 2006). In the earliest stage and under correct environmental conditions, EPEC express BFP, intimin, and the T3SS/translocon apparatus. Next, EPEC adhere to the surface of the intestinal epithelium via BFP and EspA filaments, and the T3SS injects the bacterial translocated intimin receptor (Tir) and effector proteins (EspB, EspD, EspF, EspG, and Map) directly into the host cell (Dean and Kenny 2009). The effectors activate cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli. Finally, bacteria intimately adhere to host cell by intimin-Tir interactions, causing a cytoskeletal rearrangement that result in pedestal-like structure. Tir promotes cytoskeletal reorganization through interaction with WASP (Wiskott-Aldrich syndrome protein) (Campellone et al. 2002) and other proteins, leading to the effacement of the microvilli and the production of pedestals (Goosney et al. 2001; Dean and Kenny 2009). The translocated effectors disrupt host cell processes, resulting in loss of tight-junction integrity and mitochondrial function, leading to both electrolyte loss and eventual cell death.

8 Diagnosis of EPEC Infection

Traditionally, EPEC strains were identified by O:H serotyping, but serotype designation is no longer a necessary trait for a strain to be considered EPEC. The majority of EPEC strains fall into well-established O:H serotypes (Nataro and Kaper 1998). EPEC strains often have been distinguished from other *E. coli* strains by their ability to adhere to epithelial cells in vitro in the so-called localized adherence (LA) pattern (Scaletsky et al. 1984). The fluorescent-actin-staining (FAS) assay, originally described by Knutton et al. (1989), uses fluorescein iso-thiocyanate (FITC)-or rhodamine-conjugated phaloidin to detect the actin accumulates under EPEC adhesion pedestals. Many clinical laboratories; however, do not have tissue culture facilities, and thus neither the HEp-2 assay nor the FAS test can be routinely used for diagnosis in these settings. For this reason, genotypic tests for detection of EPEC are the preferred method of identification and generally involve DNA probe hybridization or polymerase chain reaction (PCR) targeting EAF, *eae*, and *bfpA* (Nataro and Kaper 1998). A 1-kb region of the E2348/69 EAF plasmid, pMAR2, called the EAF probe,

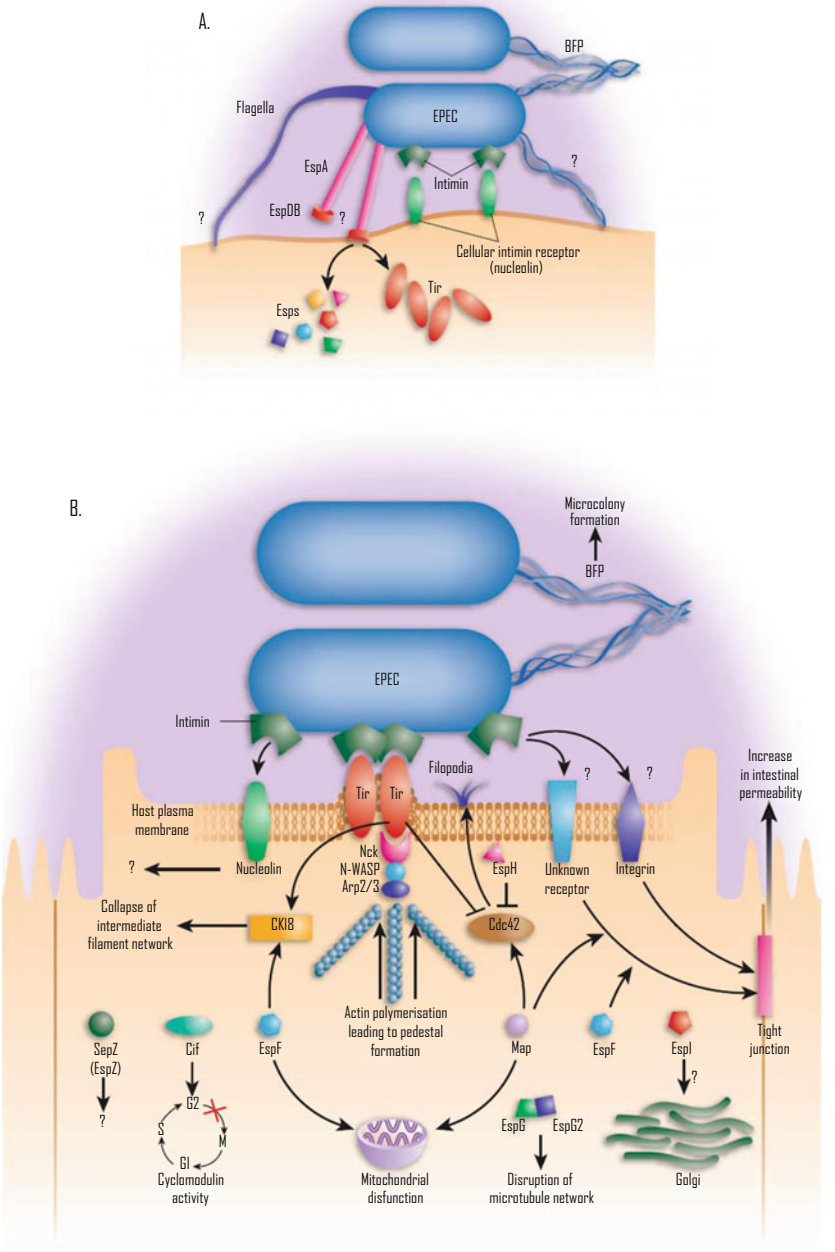

Fig. 3.2 Model of EPEC adhesion to host cells. Reprinted from Torres 2006; EcoSalPlus 1.19

was the first molecular diagnostic tool for EPEC also developed in oligonucleotide and PCR versions (Nataro and Kaper 1998). A DNA probe to detect the presence of the *eae* gene encoding intimin has been developed ((Nataro and Kaper 1998). Several probes and primers also have been developed to identify the *bfpA* gene encoding bundling (Nataro and Kaper 1998), but may fail to identify all *bfpA*-positive EPEC strains as multiple alleles of *bfpA* have been identified (Blank et al. 2000), suggesting that some current PCR methods may fail to identify all *bfpA*-positive EPEC strains.

9 Clinical Considerations of EPEC Infection

Diarrheal disease by EPEC varied from subclinical to fatal infection (Torres 2015). EPEC causes primarily acute diarrhea, although many persistent cases, lasting more than 2 weeks, have also been reported (Levine and Edelman 1984; Nataro and Kaper 1998; Fagundes-Neto and Scaletsky 2000). Recently, EPEC infection was associated with a 2.8 fold elevated risk of death among infants in Kenya (Kotloff et al. 2013).

In addition to profuse watery diarrhea, vomiting, dehydration, and low-grade fever are common symptoms of EPEC infection (Nataro and Kaper 1998; Goosney et al. 2001). Furthermore, EPEC infection may lead to severe malabsorption of nutrients, and even evolve to lactose intolerance and food allergy, resulting in nutritional aggravation and persistence of diarrhea (Fagundes-Neto and Scaletsky 2000). Edema, neutrophil infiltrate, and reduced enzymatic activity in the intestinal mucosa have been found following EPEC infection (Arenas-Hernandez et al. 2012). Correction of the fluid and electrolytes losses should be properly replaced as the first stage of the treatment. Correction of nutritional imbalance with lactose-free formula or breast milk may be insufficient for some severe cases, and total parental nutrition may be required (Fagundes-Neto and Scaletsky 2000).

Persistent infections may require the use of antimicrobials; however, multiple antibiotic resistance is common for EPEC. Antibiotic-resistant EPEC has been related by reports of multidrug-resistant EPEC strains from diverse parts of the world (Subramanian et al. 2009; Scaletsky et al. 2010; Mitra et al. 2011; Croxen et al. 2013). EPEC displays resistance to a range of antibiotics, including penicillins, cephalosporins, and aminoglycosides (Subramanian et al. 2009). A study of 149 EPEC strains isolated from children in Brazil found that resistance was more common among typical EPEC strains than among atypical EPEC strains (Scaletsky et al. 2010). In addition, a conserved conjugative plasmid carrying antibiotic resistance was identified among typical strains of serotypes O111:H2, O66:H6, O127:H6, and O119:H6 (Nwaneshiudu et al. 2007; Scaletsky et al. 2010). Other therapies such as bismuth subsalicylate, specific bovine anti-EPEC milk Igs, and also zinc (Croxen et al. 2013; Patel et al. 2010) have also proven useful.

There are no vaccines currently available to prevent disease due to EPEC. Antibodies against EPEC O antigens and outer membrane proteins have been found in breast milk (Costa-Carvalho et al. 1994; Cravioto et al. 1991), and protection from mother to infant can be transmitted through colostrum IgA (Carbonare et al. 1997; Loureiro et al. 1998). Antibodies from maternal colostrum and serum samples have been shown to recognize

BFP, intimin, EspA, and EspB (Tacket et al. 2000; Parissi-Crivelli et al. 2000. Therefore, several candidate vaccines based on EspB (Quintana Flores et al. 2002; De Souza Campos Fernandes et al. 2003), BFP (De Souza Campos Fernandes et al. 2003), and intimin (Stakenborg et al. 2006) have been explored. Recently, bacterial ghosts devoid of cytoplasmic contents but expressing all EPEC surface components were constructed and used in vaccination challenge experiments with mice (Liu et al. 2012). Vaccinated mice showed 84–90 % protection in control mice (Liu et al. 2012). Homologous rechallenge with wild-type EPEC resulted in a reduced severity of disease but had no effect on incidence of diarrhea (Donnenberg et al. 1998).

Acknowledgment My laboratory is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPES) and Conselho de Desenvolvimento Científico e Técnológico (CNPq).

References

Afset JE, Bergh K, Bevanger L (2003) High prevalence of atypical enteropathogenic *Escherichia coli* in Norwegian children with diarrhea. J Med Microbiol 52:1015–1019

Alikhani MY, Mirsalehian A, Aslani MM (2006) Detection of typical and atypical enteropathogenic *Escherichia coli* (EPEC) in Iranian children with and without diarrhea. J Med Microbiol 55:1159–1163

Arenas-Hernandez MM, Martinez-Laguna Y, Torres AG (2012) Clinical implications of enteroadherent *Escherichia coli*. Curr Gastroenterol Rep 14:386–394

Badea L, Doughty S, Nicholls L et al (2003) Contribution of Efa/LifA to the adherence of enteropathogenic *Escherichia coli* to epithelial cells. Microb Pathog 34:205–215

Bakhshi B, Fallahzad S, Pourshafie MR (2013) The occurrence of atypical enteropathogenic *Escherichia coli* strains among children with diarrhea in Iran. J Infect Chemother 19:615–620

Baldini MM, Kaper JB, Levine MM et al (1983) Plasmid mediated adhesion of enteropathogenic *Escherichia coli*. J Pediatr Gastroenterol Nutr 2:534–538

Barba J, Bustamante VH, Flores-Valdez MA et al (2005) A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. J Bacteriol 187:7918–7930

Behiry IK, Abada EA, Ahmed EA, Labeed RS (2011) Enteropathogenic *Escherichia coli* associated with diarrhea in children in Cairo, Egypt. Sci World J 11:2613–2619

Bieber D, Ramer SW, Wu CY et al (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science 280:2114–2118

Blank TE, Zhong H, Bell AL et al (2000) Molecular variation among type IV pilin (*bfpA*) genes from diverse enteropathogenic *Escherichia coli* strains. Infect Immun 68:7028–7038

Bortoloni M, Trabulsi L, Keller R et al (1999) Lack of expression of bundle-forming pili in some clinical isolates of enteropathogenic *Escherichia coli* (EPEC) is due to a conserved large deletion in the *bfp* operon. FEMS Microbiol Lett 179:169–174

Bray J (1945) Isolation of antigenically homogeneous strains of *Bacterium coli neopolitanum* from summer diarrhoea of infants. J Pathol Bacteriol 57:239–247

Brinkley C, Burland V, Keller R et al (2006) Nucleotide sequence analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid pMAR7. Infect Immun 74:5408–5413

Campellone KG, Giese A, Tipper DJ, Leong JM (2002) A tyrosine-phosphorylated 12-aminoacid sequence of enteropathogenic *Escherichia coli* Tir binds the host adaptor protein Nck and is required for Nck localization to actin pedestals. Mol Microbiol 43:1227–1241

Carbonare SB, Silva ML, Palmeira P, Carneiro-Sampaio MM (1997) Human colostrum IgA antibodies reacting to enteropathogenic *Escherichia coli* antigens and their persistence in the faeces of a breastfed infant. J Diarrhoeal Dis Res 15:53–58

Centers for Disease Control and Prevention (CDC) (2013) Surveillance for foodborne disease outbreaks-United States, 2009-2010. MMWR Morb Mortal Wkly Rep 62:41–47

Clearly J, Lai L-C, Shaw RK et al (2004) Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. Microbiology 150:527–538

Costa-Carvalho BT, Bertipaglia A, Solé D et al (1994) Detection of immunoglobulin (IgG and IgA) anti-outer membrane proteins of enteropathogenic *Escherichia coli* (EPEC) in saliva, colostrum, breast milk, serum, cord blood and amniotic fluid. Study of inhibition of localized adherence of EPEC to HeLa cells. Acta Paediatr 83:870–873

Cravioto A, Tello A, Villafan H et al (1991) Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. J Infect Dis 163:1247–1255

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26:822–880

De Souza Campos Fernandes RC, Quintana Flores VM, Sousa de Macedo Z, Medina-Acosta E (2003) Coproantibodies to the enteropathogenic *Escherichia coli* vaccine candidates BfpA and EspB in breast fed and artificially fed children. Vaccine 21:1725–1731

Dean P, Kenny B (2009) The effector repertoire of enteropathogenic *E. coli* ganging up on the host cell. Curr Opin Microbiol 12:101–109

Deng W, Puente JL, Grunheid S et al (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. Proc Natl Acad Sci U S A 101:3597–3602

DeVinney R, Knoechel DG, Finlay BB (1999) Enteropathogenic *Escherichia coli*: cellular harassment. Curr Opin Microbiol 2:83–88

Donnenberg MS, Kaper JB (1992) Enteropathogenic *Escherichia coli*. Infect Immun 60:3953–3961

Donnenberg MS, Tacket CO, Losonsky G, Frankel G, Nataro JP, Dougan G, Levine MM (1998) Effect of prior experimental human enteropathogenic *Escherichia coli* infection on illness following homologous and heterologous re-challenge. Infect Immun 66:52–58

Drago-Serrano ME, Parra SG, Manjarrez-Hernández HA (2006) EspC, an autotransporter protein secreted by enteropathogenic *Escherichia coli* (EPEC), displays protease activity on human hemoglobin. FEMS Microbiol Lett 265:35–40

Dulguer MV, Fabricotti SH, Bando SY et al (2003) Atypical enteropathogenic *Escherichia coli* strains: phenotypic and genetic profiling reveals a strong association between enteroaggregative *E. coli* heat-stable enterotoxin and diarrhea. J Infect Dis 188:1685–1694

Elliott SJ, Sperandio V, Giron JA et al (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic *Escherichia coli*. Infect Immun 68:6115–6126

Fagundes-Neto U, Freymuller GMSV, Schmitz LG, Scaletsky I (1995) Enteropathogenic *Escherichia coli* O111:H2 penetrates the small bowel epithelium in an infant with acute diarrhoea. Acta Pediatr 84:453–455

Fagundes-Neto U, Scaletsky ICA (2000) The gut at war: the consequences of enteropathogenic *Escherichia coli* infection as a factor of diarrhea and malnutrition. São Paulo Med J 118:21–29

Fletcher JN, Embaye HE, Getty B et al (1992) Novel invasion determinant of enteropathogenic *Escherichia coli* plasmid pLV501 encodes the ability to invade intestinal epithelial cells and HEp-2 cells. Infect Immun 60:2229–2236

Frankel G, Candy DCA, Everest P, Dougan G (1994) Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. Infect Immun 62:1835–1842

Franzolin MR, Alves RCB, Keller R, Gomes TAT, Beutin L, Barreto ML, Milroy C, Strina A, Ribeiro H, Trabulsi LR (2005) Prevalence of diarrheagenic *Escherichia coli* in children with diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 100:359–363

Girón JA, Ho AS, Schoolnik GK (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254(5032):710–713

Girón JA, Ho ASY, Schoolnik GK (1993) Characterization of fimbriae produced by enteropathogenic *Escherichia coli*. J Bacteriol 175:7391–7403

Girón JA, Torres AG, Freer E et al (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol Microbiol 44:361–479

Goosney DL, DeVinney R, Finlay BB (2001) Recruitment of cytoskeletal and signaling proteins to enteropathogenic and enterohemorrhagic *Escherichia coli* pedestals. Infect Immun 69:3315–3322

Hazen TH, Sahl JW, Fraser CM et al (2013) Refining the pathovar paradigm via phylogenomics of the attaching and effacing *Escherichia coli*. Proc Natl Acad Sci U S A 110:12810–12815

Hazen TH, Kaper JB, Nataro JP, Rasko DA (2015) Comparative genomics provides insight into the diversity of the attaching and effacing Escherichia coli virulence plasmids. Infect Immun 83:4103–4117

Hu J, Torres AG (2015) Enteropathogenic Escherichia coli: foe or innocent bystander? Clin Microbiol Infect 21(8):729–734

Hyland RM, Sun J, Griener TP et al (2008) The bundling pilin protein of enteropathogenic *Escherichia coli* is an N-acetyllactosamine-specific lectin. Cell Microbiol 10(1):177–187

Kenny B, DeVinney R, Stein M et al (1997) Enteropathogenic *Escherichia coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91:511–520

Klapproth JM, Scaletsky ICA, McNamara BP et al (2000) A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. Infect Immun 68(4):2148–2155

Klapproth JM, Sasaki M, Sherman M et al (2004) *Citrobacter rodentium lifA/efa1* is essential for colonic colonization and crypt cell hyperplasia *in vivo*. Infect Immun 73:1441–1451

Knutton S, Baldwin T, Williams PH, McNeish AS (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect Immun 57:1290–1298

Kotloff KL, Nataro JP, Blackwelder WC et al (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicentre Study, GEMS): a prospective, case-control study. Lancet 382:209–222

Lacher DW, Steinsland H, Whittam TS (2006) Allellic subtyping of the intimin locus (*eae*) of pathogenic *Escherichia coli* by fluorescent RFLP. FEMS Microbiol Lett 261:80–87

Lacher DW, Steinsland H, Blank TE et al (2007) Molecular evolution of typical enteropathogenic *Escherichia coli*: clonal analysis by multilocus sequence typing and virulence gene allelic profiling. J Bacteriol 189:342–350

Lara-Tejero M, Galan JE (2000) A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science 290:354–357

Levine MM, Edelman R (1984) Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol Rev 6:31–51

Levine MM, Bergquist EJ, Nalin DR et al (1978) *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat stable enterotoxins and are non-invasive. Lancet 1(8074):1119–1122

Liu J, Wang WD, Liu YJ, Liu S, Zhou B, Zhu LW, Ji X, Sun Y, Feng SZ (2012) Mice vaccinated with enteropathogenic *Escherichia coli* ghosts show significant protection against lethal challenges. Lett Appl Microbiol 54:255–262

Loureiro I, Frankel G, Adu-Bobie J et al (1998) Human colostrum contains IgA antibodies reactive to enteropathogenic *Escherichia coli* virulence-associated proteins: intimin, BfpA, EspA, and EspB. J Pediatr Gastroenterol Nutr 27:166–171

Maranhão HS, Medeiros MCC, Scaletsky ICA et al (2008) The epidemiological and clinical characteristics and nutritional development of infants with acute diarrhea, in northeastern Brazil. Ann Trop Med Parasitol 102:357–365

McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A 92:1664–1668

Mitra M, Ahmad P, Mehdi R, Hosein A, Ahmad K (2011) Multiple drug resistance of enteropathogenic *Escherichia coli* isolated from children with diarrhea in Kashan, Iran. Afr J Microbiol Res 5:3305–3309

Moon HW, Whipp SC, Argenzio RA et al (1983) Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect Immun 41:1340–1351

Moreira CG, Palmer K, Whiteley M et al (2006) Bundle-forming pili and EspA are involved in biofilm formation by enteropathogenic *Escherichia coli*. J Bacteriol 188:3952–3961

Nair GB, Ramamurthy T, Bhattacharya MK, Krishnan T, Ganguly S, Saha DR et al (2010) Emerging trends in the etiology of enteric pathogens as evidenced from an active surveillance of hospitalized diarrheal patients in Kolkata, India. Gut Pathog 2:4

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201

Nataro JP, Maher KO, Mackie P, Kaper JB (1987) Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. Infect Immun 55:2370–2377

Navarro-Garcia F, Canizales-Roman A, Sui BQ et al (2004) The serine protease motif of EspC from enteropathogenic *Escherichia coli* produces epithelial damage by a mechanism different from that of Pet toxin from enteroaggregative *E. coli*. Infect Immun 72:3609–3621

Neter E, Westphal O, Luderitz O et al (1955) Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. Pediatrics 16:801–807

Nguyen RN, Taylor LS, Tauschek M, Robins-Browne RM (2006) Atypical enteropathogenic *Escherichia coli* infection and prolonged diarrhea in children. Emerg Infect Dis 12:615–620

Nicholls L, Grant TH, Robins-Browne RM (2000) Identification of a novel genetic locus that is required for *in vitro* adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. Mol Microbiol 35(2):275–288

Nougayrède J-P, Fernades PJ, Donnenberg MS (2003) Adhesion of enteropathogenic *Escherichia coli* to host cells. Cell Microbiol 5:359–372

Nwaneshiudu AI, Mucci T, Pickard DJ et al (2007) A second large plasmid encodes conjugative transfer and antimicrobial resistance in O119:H2 and some typical O111 enteropathogenic *Escherichia coli* strains. J Bacteriol 189:6074–6079

Ochoa TJ, Contreras CA (2011) Enteropathogenic *Escherichia coli* infection in children. Curr Opin Infect Dis 24:478–483

Ochoa TJ, Barletta F, Contreras C et al (2008) New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Trans R Soc Trop Med Hyg 102:852–856

Parissi-Crivelli A, Parissi-Crivelli JM, Girón JA (2000) Recognition of enteropathogenic *Escherichia coli* virulence determinants by human colostrum and serum antibodies. J Clin Microbiol 38:2696–2700

Patel A, Mamtani M, Dibley MJ et al (2010) Therapeutic value of zinc supplementation in acute and persistent diarrhea: a systematic review. PLoS One 5, e10386

Quintana Flores VM, Campos de Souza Fernandes RC, Sousade Macedo Z, Medina-Acosta E (2002) Expression and purification of the recombinant enteropathogenic *Escherichia coli* vaccine candidates BfpA and EspB. Protein Expr Purif 25:16–22

Ratchtrachenchai O-A, Subpasu S, Hayashi H, Ba-Thein W (2004) Prevalence of childhood diarrhea associated *Escherichia coli* in Thailand. J Med Microbiol 53:237–243

Robins-Browne RM (1987) Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev Infect Dis 9:28–53

Rothbaum R, McAdams AJ, Gianella R et al (1982) A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology 83:441–454

Ruchaud-Sparagano M-H, Muhlen S, Dean P, Kenny B (2011) The enteropathogenic Escherichia coli (EPEC) Tir effector inhibits NF-kB activity by targeting TNFα receptor-associated factors. PLos Pathog 7, e1002414

Saldaña Z, Erdem AL, Schuller S et al (2009) The *Escherichia coli* common pilus and the bundle-forming pilus act in concern during the formation of localized adherence by enteropathogenic *E. coli*. J Bacteriol 191:3451–3461

Salinger N, Kokona B, Fairman R, Okeke I (2009) The plasmid encoded regulator activates factors conferring lysozyme resistance on enteropathogenic *Escherichia coli* strains. Appl Environ Microbiol 75:275–280

Santos A, Finlay BB (2015) Bringing down the host: enteropathogenic and enterohaemorrhagic *Escherichia coli* effector-mediated subversion of host innate immune pathways. Cell Microbiol 17:318–332

Scaletsky IC, Silva ML, Trabulsi LR (1984) Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect Immun 45:534–536

Scaletsky ICA, Gatti MSV, Silveira D et al (1995) Plasmid encoding for drug resistance and invasion of epithelial cells in enteropathogenic *Escherichia coli* O111:H-. Microb Pathog 18:387–399

Scaletsky ICA, Souza TB, Aranda KRS, Okeke IN (2010) Genetic elements associated with antimicrobial resistance in enteropathogenic *Escherichia coli* (EPEC) from Brazil. BMC Microbiol 10:25

Silva LEP, Souza TB, Silva NP, Scaletsky ICA (2014) Detection and genetic analysis of the enteroaggregative *Escherichia coli* heat-stable enterotoxin (EAST1) gene in clinical isolates of enteropathogenic *Escherichia coli* (EPEC) strains. BMC Microbiol 14:135

Sperandio V, Li CC, Kaper JB (2002) Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement Pathogenicity Island in enterohemorrhagic *E. coli*. Infect Immun 70:3085–3093

Spina A, Kerr K, Cormican M, Barbut F, Eigentler A, Zerva L et al (2015) Spectrum of enteropathogens detected by the FilmArray GI Panel in a multicentre study of community-acquired gastroenteritis. Clin Microbiol Infect 21:719–728

Stakenborg T, Vandekerchove D, Mariĕn J et al (2006) Protection of rabbits against enteropathogenic *Escherichia coli* (EPEC) using an intimin null mutant. BMC Vet Res 2:22

Stone KD, Zhang HZ, Carlson LK, Donnenberg MS (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. Mol Microbiol 20:325–337

Subramanian K, Selvakkumar C, Vinaykumar KS et al (2009) Tackling multiple antibiotic resistance in enteropathogenic *Escherichia coli* (EPEC) clinical isolates: a diarylheptanoid from *Alpinia officinarum* shows promising antibacterial and imunomodulatory activity against EPEC and its lipopolysaccharide-induced inflammation. Int J Antimicrob Agents 33:244–250

Tacket CO, Sztein MB, Losonsky G et al (2000) Role of EspB in experimental human enteropathogenic *Escherichia coli* infection. Infect Immun 68:3689–3695

Tatsuno I, Mundy R, Frankel G, Chong Y, Phillips AD, Torres AG, Kaper JB (2006) The *lpf* gene cluster for long polar fimbriae is not involved in adherence of enteropathogenic *Escherichia coli* or virulence of *Citrobacter rodentium*. Infect Immun 74:265–272

Teixeira NB, Rojas TCG, Silveira WD et al (2015) Genetic analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF) plasmid reveals a new deletion within the EAF probe sequence among O119 typical EPEC strains. BMC Microbiol 15:200

Tobe T, Hayashi T, Han C et al (1999) Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. Infect Immun 67:5455–5462

Torres AG (2006) Adhesins of enteropathogenic *Escherichia coli*. ASMScience/EcoSalPlus 1–19

Torres AG, Kanack KJ, Tutt CB, Popov V, Kaper JB (2004) Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. FEMS Microbiol Lett 238:333–344

Trabulsi LR, Keller R, Gomes TAT (2002) Typical and atypical enteropathogenic Escherichia coli. Emerg Infect Dis 8:508–513

Vidal JE, Navarro-Garcia F (2008) EspC translocation into epithelial cells by enteropathogenic *Escherichia coli* requires a concerted participation of type V and III systems. Cell Microbiol 10:1976–1986

Vuopio-Varkila J, Schoolnik GK (1991) Localized adherence by enteropathogenic *Escherichia coli* is an inducible phenotype associated with the expression of new outer membrane proteins. J Exp Med 174:1167–1177

Whittam TS, McGraw EA (1996) Clonal analysis of EPEC serogroups. Rev Microbiol 27:7–16

Xicohtencatl-Cortes J, Saldaña Z, Deng W et al (2010) Bacterial macroscopic ropelike fibers with cytopathic and adhesive properties. J Biol Chem 285:32336–32342

Yamamoto T, Wakisaka N, Sato F, Kato A (1997) Comparison of the nucleotide sequence of enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 genes among diarrhea-associated *Escherichia coli*. FEMS Microbiol Lett 147:89–96

Chapter 4
Atypical Enteropathogenic *Escherichia coli*

Tânia A.T. Gomes, Denise Yamamoto, Mônica A.M. Vieira, and Rodrigo T. Hernandes

Summary The atypical enteropathogenic *Escherichia coli* (EPEC), a subgroup of EPEC, has the ability to cause histopathologic attaching and effacing lesions in eukaryotic cells, but lacks the virulence-associated EPEC adherence factor plasmid. The aEPEC strains may harbor virulence markers of other Diarrheagenic *E. coli* pathogroups as well as of extraintestinal pathogenic *E. coli* strains. This observation led to the assumption that aEPEC strains comprise a very heterogeneous group with diverse additional virulence mechanisms that altogether can modulate the disease outcome or their occurrence in asymptomatic subjects. While the prevalence of typical EPEC strains has declined in the last decade in most geographic areas studied, aEPEC strains are considered emerging enteropathogens that have been detected worldwide. In addition, the detection of aEPEC in different animal species, as well as in food and environmental samples, suggests that at least some aEPEC infections may be zoonotic. Herein, we review the recent achievements in the knowledge of the virulence properties, genetic background, and epidemiology of aEPEC infections in the America. Despite the recent advances, the need of discriminating between strains that can cause diarrhea and those that promote asymptomatic infections is a current motivation for further studies in the field.

T.A.T. Gomes (✉) • D. Yamamoto • M.A.M. Vieira
Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo—Escola Paulista de Medicina, São Paulo 04023-062, Brazil
e-mail: tatg.amaral@unifesp.br; dyamamoto@unifesp.br; monica.vieira@unifesp.br

R.T. Hernandes
Departamento de Microbiologia e Imunologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu, São Paulo 18618-970, Brazil
e-mail: rthernandes@ibb.unesp.br

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_4

1 General Concepts About aEPEC

Subclassification of the enteropathogenic *E. coli* (EPEC) pathogroup as typical EPEC (tEPEC) and atypical EPEC (aEPEC) was possible after the development of molecular and cellular biology methods and of tissue culture assays that provided a great amount of information about EPEC virulence factors (Kaper et al. 2004).

A remarkable feature of tEPEC and aEPEC infections comprises the formation of a characteristic histopathological lesion known as attaching and effacing (AE). In addition, both EPEC groups lack the genes encoding Shiga toxins (Stx), found in Shiga toxin-producing *E. coli* (STEC), and the heat-labile (LT) and heat-stable (ST) toxins characteristic of enterotoxigenic *E. coli* (ETEC) (Kaper et al. 2004). The main difference between the two groups is that aEPEC lack the virulence-associated EAF (EPEC adherence factor) plasmid (pEAF), which encodes a type IV pilus known as bundle-forming pilus (BFP) (Trabulsi et al. 2002). The ideal methods to differentiate between tEPEC and aEPEC are the detection of BFP production and of the BFP-associated adherence phenotype (localized adherence), since some aEPEC strains may carry a defective *bfp* operon, resulting in positive reactions with the *bfpA* gene (Trabulsi et al. 2002; Abe et al. 2009).

Several studies conducted over the last 20 years revealed a decreasing frequency of tEPEC and the increasing rates of aEPEC in many developing countries, as reviewed in Ochoa et al. (2008), Hernandes et al. (2009), and Hu and Torres (2015). Such shift in the prevalence of tEPEC and aEPEC was observed earlier in developed countries (Trabulsi et al. 2002). However, in some less developed areas (Africa and Asia), tEPEC are still one of the most important enteropathogens (Rajendran et al. 2010; Kotloff et al. 2013; Santona et al. 2013; Ben Salem-Ben Nejma et al. 2014; Langendorf et al. 2015; Odetoyin et al. 2016).

One of the first phenotypic characteristics identified in the tEPEC infections was the ability to produce compact bacterial microcolonies on the surface of infected enterocytes. This phenotype was reproduced in assays performed in vitro, employing HeLa and HEp-2 cells with 3 h of bacteria-cell interaction, and was termed localized adherence (LA) (Trabulsi et al. 2002). As aEPEC lack BFP production, such isolates are not able to produce LA and their in vitro adherence phenotype to epithelial cells is usually defined in assays performed in extended periods (6 h) of bacteria–cell interaction (Trabulsi et al. 2002). In such assays, the adherence patterns of most aEPEC strains were classified as the localized adherence-like (LA-like) pattern, being this adherence phenotype characterized by formation of loosen microcolonies as compared to those observed in the LA pattern (Trabulsi et al. 2002). Besides LA-like, many aEPEC isolates adhere to HeLa/HEp-2 cells in undefined patterns or are non-adherent (Abe et al. 2009; Scaletsky et al. 2009; Gomes et al. 2011). In addition, some aEPEC can produce the aggregative (AA) or diffuse (DA) patterns of adherence, which comprise phenotypic markers essential for the diagnosis of enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), respectively (Vieira et al. 2001; Abe et al. 2009).

The AE lesion is characterized by bacteria intimately adhering to the epithelial cells promoting microvilli destruction and formation of pedestal-like structures that are rich in F-actin and other cytoskeletal elements (Kaper et al. 2004). The virulence factors necessary for the establishment of the AE lesion are encoded by a chromosomal pathogenicity island (PAI) called the Locus of Enterocyte Effacement (LEE) (Kaper et al. 2004). The LEE region harbors genes that encode intimin (an adhesive protein), its translocated receptor Tir (translocated intimin receptor), as well as structural components of a type III secretion system (T3SS), regulators (Ler, GrlA-Global Regulator of LEE-Activator, GrlR-Global Regulator of LEE-Repressor), translocators (EspA, EspB, EspD), chaperones (CesAB, CesD, CesD2, CesF, CesT), and effector proteins (EspG, EspZ, EspH, Map, EspF) (Croxen et al. 2013).

Ler (LEE-encoded regulator), GrlA, and GrlR are regulatory proteins encoded by genes located in the LEE region that control the expression of important LEE-virulence genes. The Ler protein is a positive regulator of the LEE genes (Kaper et al. 2004) and of genes located elsewhere, such as *nleA*, *lpf*, *and espC* (Torres et al. 2007; Roe et al. 2007). On the other hand, Ler negatively regulates several other genes like the *gfc* operon (Abe et al. 2008), which encodes proteins necessary for capsule biogenesis. The GrlA and GrlR encode proteins that regulate positively and negatively the *ler* expression, respectively (Deng et al. 2004).

Since aEPEC strains lack pEAF, many isolates are not submitted to the regulatory processes orchestrated by proteins encoded in the plasmid-encoded *perABC* operon. However, several studies have provided evidence that some aEPEC strains may possess a defective version of the pEAF (Bortolini et al. 1999; Teixeira et al. 2015). These defective plasmids usually bear a deletion in the *bfp* operon, but still harbor the *perABC* genes. In tEPEC, the PerA protein is responsible for the activation of *bpf* operon expression while PerC induces the expression of *ler*, which, in turn, activates the expression of LEE genes (Croxen et al. 2013; Wong et al. 2011). The PerC or GrlA proteins can independently activate *ler* expression and, consequently, the expression of LEE genes (Bustamante et al. 2011). Recently, a study focusing in the interaction of aEPEC with the host concluded that the absence of Per proteins in the regulatory cascade of virulence genes can produce a delay in AE lesion formation by this pathogen, and probably interferes with the ability of such strains to promote disease (Bueris et al. 2015).

By sequencing two aEPEC strains of serotypes O8:HNM and O119:H6, Gartner and colleagues demonstrated that their LEE region exhibited a genetic organization analogous to that found in the tEPEC prototype E2348/69 strain (Gärtner et al. 2004). Additionally, the authors demonstrated that genes encoding the T3SS are highly conserved, while genes encoding effector proteins show major differences. In another study, the same research group sequenced two additional strains of serotypes O26:K60 and O128:H2 and compared the data among all four strains studied (Müller et al. 2009). Altogether, they confirmed that although the LEE core is conserved, striking differences were found between the 5′ and 3′ flanking regions, probably reflecting the different lines of evolution in the aEPEC history.

For the subversion of actin dynamics, the prototype tEPEC strain E2348/69 recruits Nck to the site of bacterial adhesion in a Tir phosphorylated Y474-dependent

mechanism. Nck activates the neural Wiskott-Aldrich syndrome protein (N-WASP) triggering actin polymerization via activation of the Arp2/3 complex (Wong et al. 2011). In contrast, Tir_{EHEC} (enterohemorrhagic *E. coli* [EHEC] O157:H7) lacks an Y474 equivalent and utilizes the T3SS-translocated effector protein $EspF_U$/TccP (Tir-cytoskeleton coupling protein), which binds IRTKS/IRSp53 and the GTPase-binding domain (GBD) of N-WASP, and stimulates Nck-independent actin polymerization (Wong et al. 2011). To date, two variants of the *tccP* gene were described: *tccP* (carried on prophage CP-933U/Sp14) and *tccP2* (carried on prophage Sp4/CP-933M). It has been reported that aEPEC strains may carry *tccP* and/or *tccP2*, thus demonstrating that aEPEC strains may use both Tir-Nck and Tir-TccP pathways to induce actin polymerization (Ooka et al. 2007). Interestingly, by analyzing a collection of aEPEC, Rocha and colleagues (2011) demonstrated that one non-adherent aEPEC strain of serotype O88:HNM, when transformed with a TccP expressing-plasmid, acquired the ability to adhere to and to induce actin-accumulation in HeLa cells, an indirect measure of the ability to form AE lesions.

Intimin, a 94 kDa-outer-membrane protein, is one of the most important EPEC colonization factors, which mediates the intimate adherence to the host (Kaper et al. 2004). The polymorphism found in the C-terminal region of the *eae* gene allowed the description of at least 35 distinct intimin subtypes (Hernandes et al. 2009). Studies evaluating the prevalence of these subtypes have shown that intimins classified as beta1, epsilon1, and theta appear as the most frequent among aEPEC strains (Gomes et al. 2004; Abe et al. 2009; Contreras et al. 2010; Xu et al. 2016).

A previous study demonstrated that an aEPEC strain, carrying intimin subtype omicron, could invade epithelial cells in vitro in an intimin-dependent pathway, since an isogenic *eae* mutant was noninvasive (Hernandes et al. 2008). Further, studies pointed out that the invasive phenotype can be observed in one of three aEPEC strains harboring intimin subtype beta1 (Pacheco et al. 2014), as well as in aEPEC with uncommon intimin subtypes, such as tau and upsilon (Yamamoto et al. 2009). Of note, invasion is not considered a common virulence feature of aEPEC (Croxen et al. 2013; Pacheco et al. 2014).

Several studies have found aEPEC genes associated with adhesive structures of other DEC pathogroups as well as of extraintestinal pathogenic *E. coli*, probably suggesting that this pathogroup can employ different adherence strategies in addition to the Tir–intimin interaction (Gomes et al. 2004; Tennant et al. 2009; Scaletsky et al. 2010; Gomes et al. 2011; Hernandes et al. 2011). A summary of the adhesin-encoding genes found in aEPEC strains and their occurrence are presented in Table 4.1.

So far, the only adhesin originally described in aEPEC is the locus of diffuse adherence (LDA), an afimbrial adhesin identified in an aEPEC strain of serotype O26:H11. A cloned 15-kb genomic region from this strain confers the diffuse pattern of adherence on HEp-2 cells, when expressed in *E. coli* K-12 (Scaletsky et al. 2005). In addition to aEPEC serotype O26:H11, genes related with the *lda* operon were reported in some aEPEC of the following serotypes: O26:HND, O157:HNM, and ONT:H18 (Scaletsky et al. 2010).

Table 4.1 Adhesin-encoding genes in aEPEC isolates

Adhesin	Target	No. of aEPEC		%	References
		Studied	Positive		
Type 1 pilus	*fimH*	57	57	100.0	Afset et al. (2006)
Curli	*csgE*	57	57	100.0	Afset et al. (2006)
Hemorrhagic *coli* pilus	*hcpA*	71	70	98.6	Hernandes et al. (2011)
E. coli common pilus	*ecpA*	71	61	85.9	Hernandes et al. (2011)
E. coli laminin-binding fimbriae	*elfA*	71	51	71.8	Hernandes et al. (2011)
Long polar fimbriae	*lpfA*	100	59	59.0	Gomes et al. (2011)
Porcine A/E-associated adhesin	*paa*	100	42	42.0	Gomes et al. (2011)
EHEC factor for adherence	*efa1*/*lifA*	107	32	30.0	Vieira et al. (2010)
IrgA homologue adhesin	*Iha*	100	29	29.0	Gomes et al. (2011)
Antigen 43	*agn43*	57	12	21.1	Afset et al. (2006)
ToxB protein of EHEC	*toxB*	126	17	13.5	Scaletsky et al. (2009)
Afimbrial adhesin of the Dr family	*afaBC*	99	8	8.0	Gomes et al. (2004)
F1845	*daaC*	99	7	7.0	Gomes et al. (2004)
Locus for diffuse adherence	*ldaH*	126	6	4.8	Scaletsky et al. (2009)
P fimbriae	*papC*	99	1	1.0	Gomes et al. (2004)
S fimbriae	*sfaDE*	99	1	1.0	Gomes et al. (2004)

Little information is available regarding how the environmental intestinal conditions can influence aEPEC infection of enterocytes. In a study focused in evaluating how the intestinal environmental conditions could modulate the aEPEC interactions with the host, Romão and colleagues demonstrated that the presence of 5 % of CO_2 significantly enhanced the number of bacteria associated with the epithelial cells (Romão et al. 2014). Previous studies showed that a 5 % CO_2-containing atmosphere enhanced protein secretion by the EPEC T3SS (Haigh et al. 1995). The findings of Romão and colleagues (Romão et al. 2014) support the role of the T3SS-translocon as an additional aEPEC adhesin (Hernandes et al. 2013).

The ability of aEPEC strains to adhere to abiotic surfaces (polystyrene and glass) has also been demonstrated (Culler et al. 2014; Nascimento et al. 2014). One study employing aEPEC serotype O55:H7 implicated the non-fimbrial adhesin curli as the structure mediating binding to these surfaces at 26 °C, while the common type 1 pilus (T1P) was associated with the same phenotype in assays performed at 37 °C (Weiss-Muszkat et al. 2010; Hernandes et al. 2013).

Non-LEE effectors (Nle) are additional T3SS-dependent effector proteins encoded by genes organized outside the LEE region, in at least six chromosomal

PAIs, or in prophage elements (reviewed in Wong et al. 2011 and Vossenkämper et al. 2011). Although they are not necessary for AE lesion formation, it is believed that these effectors enhance the bacterial virulence.

Since the beginning of 2000, many Nle effectors have been identified and characterized in EHEC, EPEC, and *Citrobacter rodentium* prototype strains, with Cif (Cycle inhibiting factor) being the first Nle identified (Vossenkämper et al. 2011). After the description of the first Nle effector, many others were functionally characterized and shown to disturb host cells cytoskeleton and tight junctions and to modulate or prevent the host inflammatory response (reviewed in Vossenkämper et al. 2011; Wong et al. 2011; Raymond et al. 2013).

Until now, only two Nle effectors, Ibe and EspT, have been functionally described and characterized in aEPEC (Buss et al. 2009; Bulgin et al. 2009). Ibe was identified in aEPEC strain 3431-4 (Buss et al. 2009) as a protein that interacts with the C-terminal domain of the host protein IQGAP1 and appears to enhance membrane ruffles and N-WASP activation, which consequently increases actin polymerization and bacterial pedestal formation (Buss et al. 2009). Although the EspT virulence mechanism was identified in aEPEC strain E110019 (Bulgin et al. 2009), which was responsible for a diarrheal outbreak in Finland in 1987, this effector is not commonly found among aEPEC strains (Arbeloa et al. 2009). EspT modulates actin dynamics (Bulgin et al. 2009) leading to membrane ruffling, and has also been associated with cell invasion (Bulgin et al. 2009) and induction of macrophages to produce interleukins IL-8, IL-1β, and PGE2, via NF-kB and Erk/JNK pathways.

One of the first studies to investigate the occurrence of the T3SS-dependent non-LEE effectors genes in aEPEC demonstrated that *nleC* and *nleF* are the most frequent among the genes tested; however, a vast repertory of this class of effectors was detected in different frequencies (Afset et al. 2006). Despite the occurrence of several non-LEE effector-encoding genes, a case-control statistical analysis identified that *nleB* and *nleE* genes, which are located in a pathogenicity island termed PAI-O122, were more prevalent among aEPEC strains from patients than from controls, suggesting an association of these virulence factors with the diarrheal disease (Afset et al. 2006). Although in Brazil, the *nleB* and *nleE* genes could not be individually detected in association with diarrhea (Scaletsky et al. 2009; Vieira et al. 2010; Salvador et al. 2014), in one of these studies, aEPEC harboring a complete PAI-O122 (*efa1*/*lifA*, *sen*, *pagC*, *nleB*, and *nleE*) were observed more frequently among aEPEC strains obtained from diarrheic patients than from controls, showing a positive association with diarrhea, despite the occurrence of incomplete versions of this island in both tEPEC and aEPEC strains (Vieira et al. 2010).

The presence of six Nle-encoding genes (*cif*, *espI*/*nleA*, *nleB*, *nleC*, *nleD*, and *nleE*) was investigated among 107 EPEC strains (44 tEPEC and 63 aEPEC) isolated from 71 diarrheic and 36 non-diarrheic children in Brazil (Salvador et al. 2014). The Nle genes *nleC*, *cif*, and *nleB* were more prevalent among tEPEC than aEPEC strains; however, a larger number of different *nle*-genes combinations were observed among the aEPEC. To note, none of the Nle-encoding genes investigated were statistically associated with diarrhea (Salvador et al. 2014).

By employing phylogenetic analysis to evaluate 196 aEPEC strains from seven countries, Ingle et al. (2016) gathered bacterial strains into three distinct groups, showing a variability of Nle genes combinations among these lineages. As previously suggested, the diverse repertoire of the Nle genes found among distinct aEPEC serotypes may suggest that different isolates can employ distinct strategies to promote damage to the host and cause disease (Wong et al. 2011). The Nle effectors, functions, and occurrence among aEPEC strains are listed in Table 4.2.

The autotransporter (AT) proteins are a family of secreted proteins, which are associated with bacterial adherence, aggregation, invasion, biofilm formation, and

Table 4.2 Non-Lee-encoded effectors detected in aEPEC

Non-Lee-encoded effector	Modifications promoted in the host cell	Studies showing the distribution in aEPEC
Inhibition of cell detachment and modulation of cell death		
Cif	Blocks the cell cycle	Salvador et al. (2014), Ingle et al. (2016)
NleD	Inhibits AP-1 activation via JNK cleavage, leading to reduction of the apoptosis	Salvador et al. (2014), Ingle et al. (2016)
NleF	Binds to caspases to inhibit apoptosis	Bugarel et al. (2011), Ingle et al. (2016)
NleH1 and NleH2	Block apoptosis by Bax-inhibitor binding	Bugarel et al. (2011), Ingle et al. (2016)
EspO, EspO1, and EspO2	Impair RhoA activation by EspM2, preventing cell detachment	Ingle et al. (2016)
Modulation or prevention of the host inflammatory response		
NleE	Impairs IkB degradation to prevent NF-kB translocation to the nucleus and, consequently, impairs the production of pro-inflammatory cytokines	Vieira et al. (2010), Bugarel et al. (2011), Salvador et al. (2014), Ingle et al. (2016)
NleC	Metalloprotease that inhibits NF-kB activation	Salvador et al. (2014), Ingle et al. (2016)
NleB, NleB1, and NleB2	Inhibit NF-kB activation in response to TNF stimulus	Vieira et al. (2010), Bugarel et al. (2011), Salvador et al. (2014)
NleI/NleG and NleG2-3	Mimicry the human U-box E3 ubiquitin ligase that interacts with E2 ubiquitin, essential step of ubiquitination signaling. May be involved with immune response suppression	Bugarel et al. (2011), Ingle et al. (2016)
NleH1 and NleH2/OspG	Sequester ribosomal protein S3 (RPS3), inhibiting p65 NF-kB subunit recruitment for immune response	Bugarel et al. (2011), Ingle et al. (2016)
NleD	Contributes to inhibition of IL-8 production in vitro	Salvador et al. (2014), Ingle et al. (2016)
Inhibition of phagocytosis		
EspJ	Inhibits phagocytosis of opsonized bacteria by FcR and C3	Ingle et al. (2016)

(continued)

Table 4.2 (continued)

Non-Lee-encoded effector	Modifications promoted in the host cell	Studies showing the distribution in aEPEC
NleH1 and NleH2/OspG	Reduces phagocytosis and opsonophagocytosis	Bugarel et al. (2011), Ingle et al. (2016)
Cytoskeleton modulation		
EspG2	Disrupts microtubules, assembles a GTPase–kinase signaling complex to inhibit organelle membrane trafficking	Ingle et al. (2016)
EspL	Binds to Annexin 2 and induces F-actin aggregation at the bacterial interaction site	Ingle et al. (2016)
EspM, EspM1, EspM2, and EspM3	Activate RhoA GTPase, promoting stress fiber formation, pedestal formation, disruption of cell polarity, and cell detachment	Arbeloa et al. (2008), Ingle et al. (2016)
EspT	Activate Cdc42 and Rac1 GTPases, inducing membrane ruffles and lammelipodia, leading to bacterial internalization	Arbeloa et al. (2009), Pacheco et al. (2014)
EspV	Induces cellular rounding, leading to formation of dendrite-like structures; induces nucleus condensation	Arbeloa et al. (2011), Ingle et al, (2016)
NleA/EspI	Inhibits protein exportation from ER to Golgi and induces tight junction disruption	Salvador et al. (2014), Ingle et al. (2016)
Ibe	Regulates Tir phosphorylation, enhancing the pedestal formation	Buss et al. (2009), Liebchen et al. (2011)
TccP/EspFu	Binds to N-WASP inhibitor releasing the catalytic domain that activates the Arp2/3 complex, leading to actin recruitment underneath of adherent bacterium	Ooka et al. (2007), Whale et al. (2007), Ingle et al. (2016)
Unknown functions		
EspK		Bugarel et al. (2011), Ingle et al. (2016)
EspN		Ingle et al. (2016)
EspW		Ingle et al. (2016)

EspR, EspS, EspX, and EspY families, NleK, and OspG1 were not identified in aEPEC strains

toxicity in Gram-negative bacteria (Henderson et al. 2004). The structure of these proteins has three functional domains: the amino-terminal leader sequence, the secreted mature protein (passenger domain), and a carboxyl-terminal domain that forms a beta-barrel pore to allow secretion of the passenger protein (Henderson et al. 2004). In a recent study, the prevalence of the following AT-encoding genes was investigated in tEPEC and aEPEC strains: *aida-1*, *cah*, *eatA*, *ehaA*, *ehaB*, *ehaC*, *ehaD*, *ehaJ*, *epeA*, *espC*, *espI*, *espP*, *pet*, *pic*, *sab*, *sat*, and *tibA* (Abreu et al. 2013). The *ehaC* gene, associated with biofilm formation in EHEC strains, was the most prevalent AT-encoding gene found among aEPEC, and its occurrence was significantly higher in aEPEC than tEPEC (Abreu et al. 2013). The same study showed that other genes associated with biofilm formation in EHEC, such as *ehaA*, *ehaB*, and *ehaD*, were detected in several of the strains tested, followed by *espC*.

The EspC protein, which is secreted from bacteria by the type five secretion system (T5SS) and injected into the host cell by the T3SS, has an IgA protease-like activity and, once in the host cytoplasm, can degrade fodrin and focal adhesion protein, leading cell to death (Navarro-Garcia et al. 2014).

Among several AT virulence factor-encoding genes found in aEPEC strains (Abreu et al. 2013), the only genes that were functionally analyzed in the aEPEC virulence process were *pic* (protein involved in intestinal colonization) and *pet* (plasmid-encoded toxin). The *pic* gene was first identified in the EAEC chromosome and was later found in a high molecular weight plasmid of one aEPEC strain (Abreu et al. 2016). By employing an aEPEC mutant in the *pic* gene, this study demonstrated that Pic shows several virulence properties, such as agglutination of rabbit erythrocytes, cleavage of mucin, degradation of components of the complement system, and colonization of mice intestines with intense mucus production (Abreu et al. 2016). Additionally, it has been shown that some aEPEC strains are able to secret Pet to the extracellular environment. Pet induces cell damages comparable to those induced by this protein when produced by strains of the EAEC pathogroup, where it was originally described (Ruiz et al. 2014).

2 Recent Advances in aEPEC Research

The identification of virulence genes among aEPEC strains has demonstrated that these strains harbor virulence markers of other DEC pathogroups as well as of *E. coli* strains that cause extraintestinal disease (extraintestinal pathogenic *E. coli*). This observation led to the conclusion that aEPEC strains, in fact, can comprise a very heterogeneous group with several additional virulence mechanisms and phenotypic characteristics (Hernandes et al. 2009).

Facing this complex genetic virulence background, recent studies have tried to decipher how truly pathogenic aEPEC strains interact with and damage the host. In recent years, some advances have been made in the understanding of the initial steps of the aEPEC interaction with the host. The observation that an aEPEC isogenic intimin-mutant strain remained adherent to HeLa cells in a T3SS-dependent mechanism provided strong evidence about the contribution of the T3SS-translocon in the bacteria–cell interaction (Hernandes et al. 2013). More recently, the flagellar cap protein FliD of an aEPEC strain (serotype O51:H40) was shown to bind unknown receptors at the Caco-2 intestinal cells microvilli (Sampaio et al. 2016). In addition, an anti-FliD serum and purified FliD were able to reduce the adherence of the aEPEC strain studied as well as that of tEPEC, EHEC, and ETEC prototype strains to this cell lineage (Sampaio et al. 2016). It has also been shown that the flagellin (FliC) of another aEPEC strain (serotype O26:H11) binds fibronectin, probably reinforcing the bacterial interaction with cellular fibronectin (Moraes et al. 2015). However, the role of T3SS and flagella in the aEPEC colonization in vivo remains to be studied. Furthermore, although the prevalence of Pic in aEPEC strains is not a common finding, this AT-protein appears to mediate colonization of mice intestines (Abreu et al. 2016).

In recent years, two research groups have used advanced techniques of genomic analysis to investigate a large collection of aEPEC strains and provided information which extended the knowledge on the aEPEC genomic diversity (Hazen et al. 2013; Ingle et al. 2016). Based on whole-genome phylogenetic analysis, Hazen and colleagues (2013) observed that 35 % of the aEPEC were placed into the EPEC1, EPEC2, or EPEC4 lineages, which are distinct EPEC lineages that probably acquired the LEE region and pEAF independently. Such evidence suggested that at least some tEPEC may have lost pEAF during the transit in the host, originating aEPEC strains. In fact, studies performed in adult volunteers, trying to demonstrate the role of tEPEC as an intestinal pathogen, had previously revealed the pEAF segregation during the development of the infectious processes (Levine et al. 1985). Curiously, in a study performed by Hazen et al. (2013), some AE *E. coli* strains (including aEPEC strains) did not fit any of the known genomic AE-producing *E. coli* lineages, suggesting that these bacterial populations are closest to other *E. coli* pathogroups (Hazen et al. 2013). These unclassified aEPEC strains could represent commensal *E. coli* strains that received the LEE region by horizontal transfer along with supplementary virulence factors-encoding genes that turned them into true pathogens.

Although several advances have been made in the knowledge of the genome of aEPEC, and the role of several proteins have been reported in in vitro studies, a major difficulty faced in order to detect the truly pathogenic isolates within this heterogeneous group of strains is the absence of an animal model of diarrhea. Despite the fact that the AE lesion comprises an important step in the pathogenesis processes of aEPEC infections, the absence of animal models hampers the identification of specific genes or genes combinations that could enhance the virulence of aEPEC isolates and their ability to cause diarrheal disease.

3 Atypical EPEC in America

A comparison of the prevalence of EPEC strains among different geographic areas and periods of time has been hampered by the diverse identification techniques (serotyping, adherence patterns, and the presence of the *eae* or conserved LEE genes) employed in different studies. Variations in the features of the populations studied and lack of discrimination between tEPEC and aEPEC in some studies have also hampered such analysis.

Several studies conducted over the last 20 years in different regions of the world revealed a decreasing frequency of tEPEC and the increasing rates of aEPEC, especially in developing countries, where aEPEC has been considered an emerging pathogroup (Cohen et al. 2005; Ochoa et al. 2008; Hernandes et al. 2009). However, such increase in prevalence may also reflect the refined EPEC discrimination between tEPEC and aEPEC (Trabulsi et al. 2002; Hernandes et al. 2009; Hu and Torres 2015).

The frequency of aEPEC strains in distinct parts of the world has been previously reviewed (Ochoa et al. 2008; Hernandes et al. 2009; Hu and Torres 2015) and was updated in Table 4.3. Epidemiological studies developed in Latin America have

Table 4.3 Atypical enteropathogenic *E. coli* in humans

Country	% of aEPEC in (population studied)		References
	Diarrheic cases	Asymptomatic cases	
America			
Brazil	1.6 % (304 adults and children)	NT	Liebchen et al. (2011)
Brazil	12.1 % (200 children)	14.7 % (150 children)	Nunes et al. (2012)
Brazil	11.3 % (141 children up to 10 years old)	7.9 % (419 children up to 10 years old)	Lozer et al. (2013)
Brazil	3.2 % (400 adults and children)	NT	Assis et al. (2014)
Brazil	8.0 % (200 children <5 years)	8.5 % (200 children <5 years)	Dias et al. (2016)
Mexico	4.5 % (831 children)	NT	Patzi-Vargas et al. (2015)
Peru	5.8 % (936 children 2–12 months of age)	9.4 % (424 children 2–12 months of age)	Contreras et al. (2011)
USA	4.3 % (823 patients 0–60 years)	3.4 % (411 patients 0–60 years)	Nataro et al. (2006)
USA	3.9 % (206 children <12 years old)	NT	Foster et al. (2015)
Other countries			
China	6.1 % (1418 diarrheic patients)	2.2 % (640 healthy carriers)	Xu et al. (2016)
Germany	6.9 % (1981 diarrheic patients up to 98 years)	NT	Hardegen et al. (2010)
India	3.8 % (394 children <5 years)	5.6 % (198 children <5 years)	Rajendran et al. (2010)
India	1.0 % (296 children)	5.0 % (100 children)	Ghosh and Ali (2010)
Italy	2.5 % (160 infants mean age 272 ± 148 days)	NT	Amisano et al. (2011)
Italy, Angola, and Mozambique	Italy 7.0 % (402 children <5 years)	NT	Santona et al. (2013)
	Angola 3.7 % (270 children <5 years)		
	Mozambique 1.9 % (377 children <5 years)		
Libya	0.08 % (243 children up to 12 years)	NT	Ali et al. (2012)
Niger	0.05 % (4020 children <5 years)	NT	Langendorf et al. (2015)
Niger	1.6 % (126 children‹5 years and their mothers)	NT	Odetoyin et al. (2016)
Tunisia	2.4 % (124 children 3–60 months)	0 % (54 children 4–60 months)	Ben Salem-Ben Nejma et al. (2014)

NT not tested

shown that the scenario has changed from the 1980s, when tEPEC prevailed over aEPEC. In the United States, a recent study has reported the presence of aEPEC in diarrheic children in a frequency similar to those found in many Latin American countries (Foster et al. 2015).

Remarkably, the role of aEPEC strains in diarrheal disease is not clear because in many regions this pathogroup has been detected in similar frequencies in both diarrheic and non-diarrheic subjects (reviewed in Hernandes et al. 2009; Hu and Torres 2015; Dias et al. 2016). Barletta and colleagues (2011) observed that there are significant statistical differences between the bacterial load of EPEC isolated from stool samples in children with and without diarrhea. Another study on diarrheic children in Dhaka, Bangladesh, evaluated the amount of PCR amplicons of the *eae* gene. Although in this region stool samples frequently carried multiple enteropathogens, these results showed that the *eae* gene load was significantly higher in diarrheal cases than in controls (Taniuchi et al. 2013). The authors, then, hypothesized that such differences could determine a correlation between bacterial load and establishment of disease. Unfortunately, a similar analysis has not been performed with aEPEC strains from diarrheic and asymptomatic subjects to verify such potential correlation. Nonetheless, while tEPEC strains affect mostly very young children (up to 1 year of age), aEPEC have been found in diarrheic patients of several ages and in adult patients with HIV-AIDS (Gomes et al. 2004; Nunes et al. 2012; Lozer and Souza 2013; Assis et al. 2014; Dias et al. 2016).

The analysis of the genetic background of aEPEC strains also has indicated that subgroups of strains could be authentic pathogens with pathogenicity being determined by specific virulence genes or group of genes more frequently associated with disease. Recent genomic studies on tEPEC isolated from patients presenting different clinical symptoms have identified genes that are more frequently associated with isolates from lethal or non-lethal symptomatic patients than with isolates from asymptomatic patients (Donnenberg et al. 2015; Hazen et al. 2016). In a very recent study performed in several regions of China, the authors analyzed the distribution of intimin subtypes among aEPEC strains and found a statistically significant difference in the distribution of the intimin-encoding gene subtype β1 (*eae*-β1) between diarrheal patients and healthy carriers (Xu et al. 2016). Another case-control study, conducted in Osaka, Japan, found β1 or γ1 intimin subtypes to be significantly associated with diarrheic disease (Wang et al. 2013). However, further studies are still required for a complete understanding of whether the several virulence determinants could lead to the establishment of disease and modulate the disease severity. Furthermore, it is important to point out that host and environmental traits can interfere in the excretion of diarrheal pathogens by asymptomatic subjects. The intestinal microbiota, the mucus layer, mucosal immunity, innate immune responses, and immune status of the host are significant aspects to be considered to explain the presence of aEPEC in these individuals (reviewed in Levine and Robins-Browne 2012).

Although many studies have isolated aEPEC from acute diarrhea, a few studies have also implicated this pathogroup as cause of persistent diarrhea and some aEPEC strains have been associated to bloody diarrhea (reviewed Hernandes et al. 2009; Hu and Torres 2015). Despite the difficulty in incriminating specific strains with diarrhea,

aEPEC strains have been implicated in diarrheal outbreaks in distinct parts of the world (reviewed in Ochoa et al. 2008; Hernandes et al. 2009; Hu and Torres 2015). A widespread outbreak caused by aEPEC strains of serotype O111:H9 in Finland involved more than 600 individuals, while in an outbreak in the United States, an aEPEC strain of serotype O39:NM was recovered from more than 100 adults. aEPEC was also involved with an outbreak affecting infants at a daycare center in Japan, in which four aEPEC isolates (serotype O55:NM) with identical Pulsed Field Gel Electrophoresis patterns were detected in distinct patients (Yatsuyanagi et al. 2002). Furthermore, a waterborne diarrheal outbreak commiting teenager students in Japan was described (Yatsuyanagi et al. 2003), where seven out of 41 diarrheic patients carried aEPEC isolates of serotype ONT:H45. In addition, a recent foodborne outbreak was reported, in which an aEPEC strain of serogroup O127a:K63, which was resistant to quinolones and extended spectrum cephalosporins, affected 112 adults in China (Hao et al. 2012), and aEPEC strains of serotype O51:H7 seemed to be specific among diarrheal patients in another epidemiological study in China (Xu et al. 2016).

A large diversity of aEPEC serotypes has been described worldwide, mainly when considering nonclassical EPEC serogroups (Trabulsi et al. 2002; Hernandes et al. 2009). More than 100 different aEPEC serogroups (O types) have been identified (Hernandes et al. 2009). The O-typeable strains reported so far belong to >4200 different serotypes, including many nonmotile and H non-typeable strains (Trabulsi et al. 2002; Hernandes et al. 2009). In addition, ~81 % of the aEPEC strains belonged to nonclassical EPEC serogroups and over 20 % of strains of nonclassical EPEC serotypes are O non-typeable.

While tEPEC are rarely found in animals and their reservoirs encompass only humans (Trabulsi et al. 2002), various aEPEC strains have been isolated from several healthy and diarrheic animal species (reviewed in Hernandes et al. 2009) and from the environment. Although there is no confirmation of direct transmission from animals to humans, animal aEPEC strains belonging to serogroups implicated in human diarrhea have been detected (e.g., O26, O103, O119, O128, O142, and O157) (reviewed in Hernandes et al. 2009; Kolenda et al. 2015). In addition, serotyping and molecular methods, such as Multilocus Sequence Typing (MSLT) or Pulsed Field Gel Electrophoresis (PFGE), have shown that domestic and wild animals and the environment are potential sources of aEPEC for human infections in several regions, including many Latin American countries, the United States, and Canada (Nakazato et al. 2004; Krause et al. 2005; Morato et al. 2009; Bentancor et al. 2010; de Almeida et al. 2012; Otero et al. 2013). These findings suggest that a large variety of animal species may define important aEPEC reservoirs (Table 4.4). Therefore, it is plausible to propose that some aEPEC strains could potentially be a zoonotic pathogen.

Some more refined techniques have enabled the investigation of environmental sources such as food as being the origin of aEPEC infection in humans. By analyzing aEPEC strains isolated from diarrheic patients and raw meats in China, it was identified that some strains from both origins shared identical serotypes, were clustered together by PFGE analyses, and harbored identical *eae* alleles (Xu et al. 2016). These results suggested that raw meats are reservoirs of aEPEC presenting *eae*-θ in China and could be transmission vehicles for diarrheic diseases. Otero and colleagues

Table 4.4 Atypical Enteropathogenic *E. coli* from animals, food, and environment

Country	Sample origin (number of sample studied)	% of aEPEC in sample	References
Americas			
Argentina	Dogs (450) and cats (149) either with or without diarrhea	2.4 % in diarrheic dogs	Bentancor et al. (2007)
Argentina	Chicken-derived products (1057)	20.0 %	Alonso et al. (2012)
Brazil	Cats (300)	5.0 %	Morato et al. (2009)
Brazil	Diarrheic (65) and asymptomatic (36) dogs	50.8 % (diarrheic); 41.7 % (asymptomatic)	de Almeida et al. (2012)
Brazil	Different free-ranging wild mammals (36)	19.4 %	Iovine Rde et al. (2015)
Canada	Wild animals (593)	10.1 %	Chandran and Mazumder (2013)
Canada	Foods, animals at slaughter, and retail meats (450)	4.0 %	Comery et al. (2013)
Canada	Forest-dominated watersheds	26.4 %	Chandran and Mazumder (2015)
USA-Mexico Border	Domestic dogs (358) and coyotes (103)	3.6 % dogs; 4.9 % coyotes	Jay-Russell et al. (2014)
USA	Norway rats from five sites in New York city (133)	38.0 %	Firth et al. (2014)
USA	Dairy cattle (100)	32.0 %	Singh et al. (2015)
Other countries			
Bangladesh	46 different natural aquatic locations (552)	13.8 %	Akter et al. (2013)
Belgium	Free-ranging wild cervids (133)	3.8 %	Bardiau et al. (2010)
France	Cattle slaughtered in six French abattoirs (1318)	4.8 %	Bibbal et al. (2015)
France	French shellfish-harvesting areas and their watersheds (English Channel coast) (505)	13.8 %	Balière et al. (2015)
Ireland	Bovine, farm soils, hide, and carcass (2700)	5.2 %	Monaghan et al. (2013)
Spain	Bulk tank milk (388)	14.7 %	Otero et al. (2013)

(2013) obtained evidence that aEPEC from ewes' milk and contaminated water presented phylogenetic relationships with human isolates, as detected by MSLT and PFGE, and therefore could be the source of human infections in farms in Spain.

Numerous aEPEC strains have also been recovered from pasteurized milk, meat samples, and vegetables (Table 4.4), indicating that animal and human stools, carrying aEPEC, contributed to the dissemination of aEPEC strains to the environment.

Despite the recent advances in the knowledge of the genetic background and pathogenicity of aEPEC, and the information generated by epidemiological studies conducted mainly in American countries, the need of discriminating between strains that can cause diarrhea and those that promote asymptomatic infection is a current motivation for further studies in the field.

Acknowledgements Work in the laboratories of T. A. T. Gomes and R. T. Hernandes is supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Abe H, Miyahara A, Oshima T et al (2008) Global regulation by horizontally transferred regulators establishes the pathogenicity of *Escherichia coli*. DNA Res 15:13–23

Abe CM, Trabulsi LR, Blanco J et al (2009) Virulence features of atypical enteropathogenic *Escherichia coli* identified by the *eae*+ EAF-negative *stx*- genetic profile. Diagn Microbiol Infect Dis 64:357–365

Abreu AG, Bueris V, Porangaba TM et al (2013) Autotransporter protein-encoding genes of diarrheagenic *Escherichia coli* are found in both typical and atypical enteropathogenic *E. coli* strains. Appl Environ Microbiol 79:411–414

Abreu AG, Abe CM, Nune KO et al (2016) The serine protease Pic as a virulence factor of atypical enteropathogenic *Escherichia coli*. Gut Microbes 0976:1–11

Afset JE, Bruant G, Brousseau R et al (2006) Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. J Clin Microbiol 44:3703–3711

Akter S, Islam M, Afreen KS et al (2013) Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh. Epidemiol Infect 141:2516–2525

Ali MMM, Mohamed ZK, Klena JD et al (2012) Molecular characterization of diarrheagenic *Escherichia coli* from Libya. Am J Trop Med Hyg 86:866–871

Alonso MZ, Lucchesi PMA, Rodríguez EM et al (2012) Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli* (STEC) in broiler chickens and derived products at different retail stores. Food Control 23:351–355

Amisano G, Fornasero S, Migliaretti G et al (2011) Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in north-west Italy. New Microbiol 34:45–51

Arbeloa A, Bulgin RR, MacKenzie G et al (2008) Subversion of actin dynamics by EspM effectors of attaching and effacing bacterial pathogens. Cell Microbiol 10:1429–1441

Arbeloa A, Blanco M, Moreira FC et al (2009) Distribution of *espM* and *espT* among enteropathogenic and enterohaemorrhagic *Escherichia coli*. J Med Microbiol 58:988–995

Arbeloa A, Oates CV, Marchès O et al (2011) Enteropathogenic and enterohemorrhagic *Escherichia coli* type III secretion effector EspV induces radical morphological changes in eukaryotic cells. Infect Immun 79:1067–1076

Assis FEA, Wolf S, Surek M et al (2014) Impact of *Aeromonas* and diarrheagenic *Escherichia coli* screening in patients with diarrhea in Paraná, Southern Brazil. J Infect Dev Ctries 8:1609–1614

Balière C, Rincé A, Blanco J et al (2015) Prevalence and characterization of Shiga toxin-producing and enteropathogenic *Escherichia coli* in shellfish-harvesting areas and their watersheds. Front Microbiol 6:1–15

Bardiau M, Gregoire F, Muylaert A et al (2010) Enteropathogenic (EPEC), enterohaemorragic (EHEC) and verotoxigenic (VTEC) *Escherichia coli* in wild cervids. J Appl Microbiol 109:2214–2222

Barletta F, Ochoa TJ, Mercado E et al (2011) Quantitative real-time polymerase Chain reaction for enteropathogenic *Escherichia coli*: a tool for investigation of asymptomatic versus symptomatic infections. Clin Infect Dis 53:1223–1229

Ben Salem-Ben Nejma I, Hassine Zaafrane M, Hassine F et al (2014) Etiology of acute diarrhea in Tunisian children with emphasis on diarrheagenic *Escherichia coli*: prevalence and identification of *E. coli* virulence markers. Iran J Public Health 43:947–960

Bentancor A, Rumi MV, Gentilini MV et al (2007) Shiga toxin-producing and attaching and effacing *Escherichia coli* in cats and dogs in a high hemolytic uremic syndrome incidence region in Argentina. FEMS Microbiol Lett 267:251–256

Bentancor A, Vilte DA, Rumi MV et al (2010) Characterization of non-Shiga-toxin-producing *Escherichia coli* O157 strains isolated from dogs. Rev Argent Microbiol 42:46–48

Bibbal D, Loukiadis E, Kérourédan M et al (2015) Prevalence of carriage of shiga toxin-producing *Escherichia coli* serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among slaughtered adult cattle in France. Appl Environ Microbiol 81:1397–1405

Bortolini MR, Trabulsi LR, Keller R et al (1999) Lack of expression of bundle-forming pili in some clinical isolates of enteropathogenic *Escherichia coli* (EPEC) is due to a conserved large deletion in the *bfp* operon. FEMS Microbiol Lett 179:169–174

Bueris V, Huerta-Cantillo J, Navarro-Garcia F et al (2015) Late establishment of the attaching and effacing lesion caused by atypical enteropathogenic *Escherichia coli* depends on protein expression regulated by Per. Infect Immun 83:379–388

Bugarel M, Martin A, Fach P, Beutin L (2011) Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains: a basis for molecular risk assessment of typical and atypical EPEC strains. BMC Microbiol 11:142

Bulgin R, Arbeloa A, Goulding D et al (2009) The T3SS effector EspT defines a new category of invasive enteropathogenic *E. coli* (EPEC) which form intracellular actin pedestals. PLoS Pathog 5, e1000683

Buss C, Müller D, Rüter C et al (2009) Identification and characterization of Ibe, a novel type III effector protein of A/E pathogens targeting human IQGAP1. Cell Microbiol 11:661–677

Bustamante VH, Villalba MI, García-Angulo VA et al (2011) PerC and GrlA independently regulate Ler expression in enteropathogenic *Escherichia coli*. Mol Microbiol 82:398–415

Chandran A, Mazumder A (2013) Prevalence of diarrhea-associated virulence genes and genetic diversity in *Escherichia coli* isolates from fecal material of various animal hosts. Appl Environ Microbiol 79:7371–7380

Chandran A, Mazumder A (2015) Pathogenic potential, genetic diversity, and population structure of *Escherichia coli* strains isolated from a forest-dominated watershed (Comox Lake) in British Columbia, Canada. Appl Environ Microbiol 81:1788–1798

Cohen MB, Nataro JP, Bernstein DI et al (2005) Prevalence of diarrheagenic *Escherichia coli* in acute childhood enteritis: A prospective controlled study. J Pediatr 146:54–61

Comery R, Thanabalasuriar A, Garneau P et al (2013) Identification of potentially diarrheagenic atypical enteropathogenic *Escherichia coli* strains present in Canadian food animals at slaughter and in retail meats. Appl Environ Microbiol 79:3892–3896

Contreras CA, Ochoa TJ, Lacher DW et al (2010) Allelic variability of critical virulence genes (*eae*, *bfpA* and *perA*) in typical and atypical enteropathogenic *Escherichia coli* in Peruvian children. J Med Microbiol 59:25–31

Croxen MA, Law RJ, Scholz R et al (2013) Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26:822–880

Culler HF, Mota CM, Abe CM et al (2014) Atypical enteropathogenic *Escherichia coli* strains form biofilm on abiotic surfaces regardless of their adherence pattern on cultured epithelial cells. Biomed Res Int 2014:10–15

de Almeida PMP, Arais LR, Andrade JRC et al (2012) Characterization of atypical Enteropathogenic *Escherichia coli* (aEPEC) isolated from dogs. Vet Microbiol 158:420–424

Deng W, Puente JL, Gruenheid S et al (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. Proc Natl Acad Sci 101:3597–3602
Dias RCB, Santos BCDOS, Santos LFDOS et al (2016) Diarrheagenic *Escherichia coli* pathotypes investigation revealed atypical enteropathogenic *E. coli* as putative emerging diarrheal agents in children living in Botucatu, São Paulo State, Brazil. APMIS 124:299–308
Donnenberg MS, Hazen TH, Farag TH et al (2015) Bacterial factors associated with lethal outcome of enteropathogenic *Escherichia coli* infection: genomic case-control studies. PLoS Negl Trop Dis 9, e0003791
Firth C, Bhat M, Firth MA et al (2014) Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *rattus norvegicus* in New York city. MBio 5:1–16
Foster MA, Iqbal J, Zhang C et al (2015) Enteropathogenic and enteroaggregative *E. coli* in stools of children with acute gastroenteritis in Davidson County, Tennessee. Diagn Microbiol Infect Dis 83:319–324
Gärtner JF, Schmidt MA, Ga JF (2004) Comparative analysis of locus of enterocyte effacement pathogenicity islands of atypical enteropathogenic *Escherichia coli*. Society 72:6722–6728
Ghosh PK, Ali A (2010) Isolation of atypical enteropathogenic *Escherichia coli* from children with and without diarrhoea in Delhi and the National Capital Region, India. J Med Microbiol 59:1156–1162
Gomes TAT, Irino K, Girão DM et al (2004) Emerging enteropathogenic *Escherichia coli* strains? Emerg Infect Dis 10:1851–1855
Gomes TAT, Hernandes RT, Torres AG et al (2011) Adhesin-encoding genes from Shiga toxin-producing *Escherichia coli* are more prevalent in atypical than in typical enteropathogenic *E. coli*. J Clin Microbiol 49:3334–3337
Haigh R, Baldwin T, Knutton S, Williams PH (1995) Carbon dioxide regulated secretion of the EaeB protein of enteropathogenic *Escherichia coli*. FEMS Microbiol Lett 129:63–67
Hao R, Qiu S, Wang Y et al (2012) Quinolone-resistant *Escherichia coli* O127a:K63 serotype with an extended-spectrum-beta-lactamase phenotype from a food poisoning outbreak in China. J Clin Microbiol 50:2450–2451
Hardegen C, Messler S, Henrich B et al (2010) A set of novel multiplex Taqman real-time PCRs for the detection of diarrhoeagenic *Escherichia coli* and its use in determining the prevalence of EPEC and EAEC in a university hospital. Ann Clin Microbiol Antimicrob 9:5
Hazen TH, Sahl JW, Fraser CM et al (2013) Refining the pathovar paradigm via phylogenomics of the attaching and effacing *Escherichia coli*. Proc Natl Acad Sci U S A 110:12810–12815
Hazen TH, Donnenberg MS, Panchalingam S et al (2016) Genomic diversity of EPEC associated with clinical presentations of differing severity. Nat Microbiol 1:15014
Henderson IR, Navarro-Garcia F, Desvaux M et al (2004) Type V protein secretion pathway : the autotransporter story. Microbiol Mol Biol Rev 68:692–744
Hernandes RT, Silva RM, Carneiro SM et al (2008) The localized adherence pattern of an atypical enteropathogenic *Escherichia coli* is mediated by intimin omicron and unexpectedly promotes HeLa cell invasion. Cell Microbiol 10:415–425
Hernandes RT, Elias WP, Vieira MAM, Gomes TAT (2009) An overview of atypical enteropathogenic *Escherichia coli*. FEMS Microbiol Lett 297:137–149
Hernandes RT, Velsko I, Sampaio SCF et al (2011) Fimbrial adhesins produced by atypical enteropathogenic *Escherichia coli* strains. Appl Environ Microbiol 77:8391–8399
Hernandes RT, De la Cruz MA, Yamamoto D et al (2013) Dissection of the role of pili and type 2 and 3 secretion systems in adherence and biofilm formation of an atypical enteropathogenic *Escherichia coli* strain. Infect Immun 81:3793–3802
Hu J, Torres AG (2015) Enteropathogenic *Escherichia coli*: foe or innocent bystander? Clin Microbiol Infect 21:729–734
Ingle DJ, Tauschek M, Edwards DJ et al (2016) Evolution of atypical enteropathogenic *E. coli* by repeated acquisition of LEE pathogenicity island variants. Nat Microbiol 1:15010
Iovine Rde O, Dejuste C, Miranda F et al (2015) Isolation of *Escherichia coli* and *Salmonella* spp. from free-ranging wild animals. Braz J Microbiol 46:1257–1263

Jay-Russell MT, Hake AF, Bengson Y et al (2014) Prevalence and characterization of *Escherichia coli* and *Salmonella* strains isolated from stray dog and coyote feces in a major leafy greens production region at the United States-Mexico border. PLoS One 9, e113433

Kaper JB, Nataro JP, Mobley HLT (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140

Kolenda R, Burdukiewicz M, Schierack P (2015) A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic E. coli. Front Cell Infect Microbiol 5:23

Kotloff KL, Nataro JP, Blackwelder WC et al (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382:209–222

Krause G, Zimmermann S, Beutin L (2005) Investigation of domestic animals and pets as a reservoir for intimin- (*eae*) gene positive *Escherichia coli* types. Vet Microbiol 106:87–95

Langendorf C, Le Hello S, Moumouni A et al (2015) Enteric bacterial pathogens in children with diarrhea in niger: diversity and antimicrobial resistance. PLoS One 10:1–18

Levine MM, Robins-Browne RM (2012) Factors that explain excretion of enteric pathogens by persons without diarrhea. Clin Infect Dis 55:303–311

Levine MM, Nataro JP, Karch H et al (1985) The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis 152:550–559

Liebchen A, Benz I, Mellmann A et al (2011) Characterization of *Escherichia coli* strains isolated from patients with diarrhea in Sao Paulo, Brazil: identification of intermediate virulence factor profiles by multiplex PCR. J Clin Microbiol 49:2274–2278

Lozer D, Souza T (2013) Genotypic and phenotypic analysis of diarrheagenic *Escherichia coli* strains isolated from Brazilian children living in low socioeconomic level communities. BMC Infect Dis 13:418

Monaghan A, Byrne B, Fanning S et al (2013) Serotypes and virulence profiles of atypical enteropathogenic *Escherichia coli* (EPEC) isolated from bovine farms and abattoirs. J Appl Microbiol 114:595–603

Moraes CTP, Polatto JM, Rossato SS et al (2015) Flagellin and GroEL mediates in vitro binding of an atypical enteropathogenic *Escherichia coli* to cellular fibronectin. BMC Microbiol 15:278

Morato EP, Leomil L, Beutin L et al (2009) Domestic cats constitute a natural reservoir of human enteropathogenic *Escherichia coli* types. Zoonoses Public Health 56:229–237

Müller D, Benz I, Liebchen A et al (2009) Comparative analysis of the locus of enterocyte effacement and its flanking regions. Infect Immun 77:3501–3513

Nakazato G, Gyles C, Ziebell K et al (2004) Attaching and effacing *Escherichia coli* isolated from dogs in Brazil: characteristics and serotypic relationship to human enteropathogenic *E. coli* (EPEC). Vet Microbiol 101:269–277

Nascimento HH, Silva LEP, Souza RT et al (2014) Phenotypic and genotypic characteristics associated with biofilm formation in clinical isolates of atypical enteropathogenic *Escherichia coli* (aEPEC) strains. BMC Microbiol 14:184

Nataro JP, Mai V, Johnson J et al (2006) Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven, Connecticut. Clin Infect Dis 43:402–407

Navarro-Garcia F, Serapio-Palacios A, Vidal JE et al (2014) EspC promotes epithelial cell detachment by enteropathogenic *Escherichia coli* via sequential cleavages of a cytoskeletal protein and then focal adhesion proteins. Infect Immun 82:2255–2265

Nunes M do RCM, Magalhães PP, Macêdo A da S, et al (2012) Attaching and effacing Escherichia coli and Shiga toxin-producing E. coli in children with acute diarrhoea and controls in Teresina/PI, Brazil. Trans R Soc Trop Med Hyg 106:43–47

Ochoa TJ, Barletta F, Contreras C, Mercado E (2008) New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. Trans R Soc Trop Med Hyg 102:852–856

Odetoyin B, Hofmann J, Aboderin A, Okeke I (2016) Diarrhoeagenic *Escherichia coli* in mother-child Pairs in Ile-Ife, South Western Nigeria. BMC Infect Dis 16:28

Ooka T, Vieira MAM, Ogura Y et al (2007) Characterization of tccP2 carried by atypical enteropathogenic *Escherichia coli*. FEMS Microbiol Lett 271:126–135

Otero V, Rodríguez-Calleja JM, Otero A et al (2013) Genetic characterization of atypical enteropathogenic *Escherichia coli* isolates from ewes' milk, sheep farm environments, and humans by multilocus sequence typing and pulsed-field gel electrophoresis. Appl Environ Microbiol 79:5864–5869

Pacheco VCR, Yamamoto D, Abe CM et al (2014) Invasion of differentiated intestinal Caco-2 cells is a sporadic property among atypical enteropathogenic *Escherichia coli* strains carrying common intimin subtypes. Pathog Dis 70:167–175

Patzi-Vargas S, Zaidi MB, Perez-Martinez I et al (2015) Diarrheagenic *Escherichia coli* carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis 9:1–18

Rajendran P, Ajjampur SSR, Chidambaram D et al (2010) Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India. Diagn Microbiol Infect Dis 68:117–122

Raymond B, Young JC, Pallett M et al (2013) Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors. Trends Microbiol 21:430–441

Rocha SPD, Abe CM, Sperandio V et al (2011) Atypical enteropathogenic *Escherichia coli* that contains functional locus of enterocyte effacement genes can be attaching-and-effacing negative in cultured epithelial cells. Infect Immun 79:1833–1841

Roe AJ, Tysall L, Dransfield T et al (2007) Analysis of the expression, regulation and export of NleA-E in *Escherichia coli* O157: H7. Microbiology 153:1350–1360

Romão FT, Hernandes RT, Yamamoto D et al (2014) Influence of environmental factors in the adherence of an atypical enteropathogenic *Escherichia coli* strain to epithelial cells. BMC Microbiol 14:299

Ruiz RC, Melo KCM, Rossato SS et al (2014) Atypical enteropathogenic *Escherichia coli* secretes plasmid encoded toxin. Biomed Res Int 2014:896235

Salvador FA, Hernandes RT, Vieira MAM et al (2014) Distribution of non-LEE-encoded type 3 secretion system dependent effectors in enteropathogenic *Escherichia coli*. Braz J Microbiol 45:851–855

Sampaio SCF, Luiz WB, Vieira MAM et al (2016) Flagellar cap protein FliD mediates adherence of atypical enteropathogenic *Escherichia coli* to enterocyte microvilli. Infect Immun 84:1112–1122

Santona S, Diaz N, Fiori PL et al (2013) Genotypic and phenotypic features of enteropathogenic *Escherichia coli* isolated in industrialized and developing countries. J Infect Dev Ctries 7:214–219

Scaletsky IC, Michalski J, Torres AG, Dulguer MV, Kaper JB (2005) Identification and characterization of the locus for diffuse adherence, which encodes a novel afimbrial adhesin found in atypical enteropathogenic *Escherichia coli*. Infect Immun 73:4753–4765

Scaletsky ICA, Aranda KRS, Souza TB et al (2009) Evidence of pathogenic subgroups among atypical enteropathogenic *Escherichia coli* strains. J Clin Microbiol 47:3756–3759

Scaletsky ICA, Aranda KRS, Souza TB, Silva NP (2010) Adherence factors in atypical enteropathogenic *Escherichia coli* strains expressing the localized adherence-like pattern in HEp-2 cells. J Clin Microbiol 48:302–306

Singh P, Sha Q, Lacher DW et al (2015) Characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. Front Cell Infect Microbiol 5:29

Taniuchi M, Sobuz SU, Begum S et al (2013) Etiology of diarrhea in Bangladeshi infants in the first year of life analyzed using molecular methods. J Infect Dis 208:1794–1802

Teixeira NB, Rojas TCG, da Silveira WD et al (2015) Genetic analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF) plasmid reveals a new deletion within the EAF probe sequence among O119 typical EPEC strains. BMC Microbiol 15:200

Tennant SM, Tauschek M, Azzopardi K et al (2009) Characterisation of atypical enteropathogenic *E. coli* strains of clinical origin. BMC Microbiol 9:117

Torres AG, López-Sánchez GN, Milflores-Flores L et al (2007) Ler and H-NS, regulators controlling expression of the long polar fimbriae of *Escherichia coli* O157:H7. J Bacteriol 189:5916–5928

Trabulsi LR, Keller R, Gomes TAT (2002) Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis 8:508–513

Vieira MA, Andrade JR, Trabulsi LR et al (2001) Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry EAE and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. J Infect Dis 183:762–772

Vieira MAM, Salvador FA, Silva RM et al (2010) Prevalence and characteristics of the O122 pathogenicity island in typical and atypical enteropathogenic *Escherichia coli* strains. J Clin Microbiol 48:1452–1455

Vossenkämper A, MacDonald TT, Marchès O et al (2011) Always one step ahead: how pathogenic bacteria use the type III secretion system to manipulate the intestinal mucosal immune system. J Inflamm 8:11

Wang L, Wakushima M, Aota T et al (2013) Specific properties of enteropathogenic *Escherichia coli* isolates from diarrheal patients and comparison to strains from foods and fecal specimens from cattle, swine, and healthy carriers in Osaka city, Japan. Appl Environ Microbiol 79:1232–1240

Weiss-Muszkat M, Shakh D, Zhou Y et al (2010) Biofilm formation by and multicellular behavior of *Escherichia coli* O55:H7, an atypical enteropathogenic strain. Appl Environ Microbiol 76:1545–1554

Whale AD, Hernandes RT, Ooka T et al (2007) TccP2-mediated subversion of actin dynamics by EPEC 2—a distinct evolutionary lineage of enteropathogenic *Escherichia coli*. Microbiology 153:1743–1755

Wong ARC, Pearson JS, Bright MD et al (2011) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. Mol Microbiol 80:1420–1438

Xu Y, Bai X, Zhao A et al (2016) Genetic diversity of intimin gene of atypical enteropathogenic *Escherichia coli* isolated from human, animals and raw meats in China. PLoS One 11:e0152571

Yamamoto D, Hernandes RT, Blanco M et al (2009) Invasiveness as a putative additional virulence mechanism of some atypical enteropathogenic *Escherichia coli* strains with different uncommon intimin types. BMC Microbiol 9:146

Yatsuyanagi J, Saito S, Sato H et al (2002) Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. J Clin Microbiol 40:294–297

Yatsuyanagi J, Saito S, Miyajima Y et al (2003) Characterization of atypical enteropathogenic *Escherichia coli* strains harboring the *astA* gene that were associated with a waterborne outbreak of diarrhea in Japan. J Clin Microbiol 41:2033–2039

Chapter 5
Enterohemorrhagic (Shiga Toxin-Producing) *Escherichia coli*

Marta Rivas, Isabel Chinen, and Beatriz E.C. Guth

Summary Enterohemorrhagic (Shiga toxin-producing) *Escherichia coli* (EHEC/STEC) is a zoonotic food- and waterborne pathogen that can cause human infections ranging from asymptomatic carriage or mild diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The isolates belong to a large number of O:H serotypes, and O157:H7 is the most prevalent serotype associated with large outbreaks and sporadic cases of HC and HUS in many countries. Advances on the knowledge of microbial pathogenesis, pathophysiology of the associated diseases, epidemiology, and risk factors have contributed to the development of several strategies trying to prevent food and environment contamination, and consequently transmission to humans. However, prevention of EHEC (STEC) infection has been difficult because of the broad spectrum of contaminated sources and the limited effectiveness of the different interventions used. The availability of effective vaccines to reduce carriage in livestock as well as for preventing human disease is a pending challenge. Specific targeted therapies against this pathogen group are another area of concern. A new risk scenario has emerged in the last decades due to the bacterial evolution that gave rise to the emergence of hypervirulent O157 clones with a worldwide distribution and other EHEC (STEC) strains with unusual combinations of pathogenic features, such as the O104:H4 strain. Because of the severity and the long-term sequelae of EHEC (STEC)-associated illnesses, they have a high social and economic cost for both the affected families and the health system. Therefore, all efforts should be directed to reduce the burden of these diseases.

M. Rivas (✉) • I. Chinen
Servicio Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas—ANLIS "Dr. Carlos G. Malbrán", Av. Vélez Sarsfield 563 (1281), Buenos Aires, Argentina
e-mail: mrivas@anlis.gov.ar; ichinen@anlis.gov.ar

B.E.C. Guth
Department of Microbiology, Immunology, and Parasitology, Universidade Federal de São Paulo, Rua Botucatu, 862, 3o. Andar Vila Clementino, São Paulo, São Paulo, Brazil
e-mail: bec.guth@unifesp.br

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_5

1 General Concepts About EHEC (STEC)

Enterohemorrhagic (Shiga toxin-producing) *Escherichia coli* (EHEC/STEC) comprise a group of zoonotic food- and waterborne pathogens whose hallmark is the ability to produce one or more cytotoxins of the Shiga toxin (Stx) family (Melton-Celsa 2014). The clinical manifestations related to EHEC (STEC) infections can range from symptom-free carriage or mild diarrhea to more severe clinical presentations like hemorrhagic colitis (HC) and a life-threatening syndrome known as Hemolytic Uremic Syndrome (HUS), affecting mainly infants and children (Tarr et al. 2005). Although the incidence of EHEC (STEC) infections varies over the world, the importance and impact of HC and HUS outbreaks on public health is enormous, being responsible as the main cause of acute renal failure in children in many countries (Tarr et al. 2005; Rivas et al. 2006a).

HUS was originally defined as a combination of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Recently, the definition of HUS has come to include documented hemolysis rather than anemia, platelet consumption rather than thrombocytopenia, and signs of renal damage rather than renal failure (Ardissino et al. 2014). There is no specific treatment for HUS, and patients are generally given supportive care for water imbalance, anemia, hypertension, and renal failure. The frequency of patients dying during the acute phase of disease is still 1–2 % (Mele et al. 2014), and almost 30 % of patients develop long-term renal damage (Spinale et al. 2013).

The discovery and history of the emergence of this *E. coli* pathotype had recently been reviewed (Kaper and O'Brien 2014). It is fascinating that more than 30 years after its first association with a human disease, our knowledge on EHEC (STEC) epidemiology, virulence properties, pathogenesis, host interactions, and molecular evolution is continuously evolving.

Escherichia coli O157:H7, linked to the first outbreak of HC in the United States in 1982 (Riley et al. 1983) and responsible for outbreaks and sporadic cases of HUS in several countries (Rivas et al. 2006a; de Souza et al. 2011; Vally et al. 2012; Terajima et al. 2014), is the prototype of this group of pathogens originally named enterohemorrhagic *E. coli* (EHEC), and nowadays still represents one of the most important and prevalent serotypes responsible for the more severe cases of disease worldwide. However, knowledge that more than 400 *E. coli* serotypes can harbor *stx* genes has led to a more general classification of the group as Shiga toxin-producing *E. coli* (STEC), but epidemiological studies carried out all over the world have demonstrated that only a proportion of them have been implicated in human disease. In addition to O157:H7, some other serogroups as O26, O45, O103, O111, O121, and O145 have been recognized as responsible for the majority of the serious cases of infection (Gould et al. 2013; Terajima et al. 2014).

In EHEC (STEC) infections, the most significant virulence factors are the Shiga toxins (Stx). Stx family comprises several toxins, related to Stx from *Shigella dysenteriae*, sharing similar structure and biological activity. Another common charac-

teristic present among these toxins is that the *stx* operon is usually found within the sequence for an inducible, lysogenic, λ-like bacteriophage (Melton-Celsa 2014). Albeit these similarities, a high degree of diversity has been identified among these proteins, and therefore, in *E. coli* two major toxin subfamilies Stx1 and Stx2 are classified, and each is composed by several variants. Although the nomenclature of these variants was rather confusing as several systems had been proposed, a consensus has been reached in more recent years and a sequence-based nomenclature has been developed for detection and subtyping of *stx* genes (Scheutz et al. 2012). According to this scheme, members of the Stx1 subfamily include Stx1a, Stx1c, and Stx1d; whereas Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g belong to the Stx2 group. More than sequence differences, it has been recognized that some of the variants are of clinical relevance as they have been associated with more severe cases such as HC and HUS, while others have been related to uncomplicated diarrhea or are probably not produced by strains causing human disease. In this respect, those producing Stx2a, Stx2c, or Stx2d have been reported to be more pathogenic than those strains producing Stx1 variants alone or both Stx1 and Stx2 (Scheutz 2014). On the other hand, the variants Stx2e, Stx2f, and Stx2g are rarely involved in human disease. In fact, Stx2e is responsible to cause edema disease of swine, a serious neurological disorder that is frequently fatal, whereas Stx2g and Stx2f have been mainly detected in animal reservoirs (Persad and LeJeune 2014).

A considerable amount of data exists to explain the stronger association of Stx2 with more severe diseases. Earlier studies had proposed differences in cytotoxicity among Stx1 and Stx2. It has been observed that while Stx1 is more toxic to mammalian cell lines, such as Vero cells, Stx2 is more potent in animal models. It had been shown that only Stx2-treated mice developed renal complications and death, and similar observations were seen in nonhuman primate models (Baboons) in which treatment with Stx2 caused HUS, while equal doses of Stx1 had no effect (Siegler et al. 2003). Moreover, comparison of the effects of the two toxins also showed different pro-inflammatory responses and different timings of organ injury. Stx1 induced a stronger and earlier pro-inflammatory response in baboons, while the Stx2 response was gradual and delayed by several days (Stearns-Kurosawa et al. 2010). Another study, also using a nonhuman primate model, showed that both Stx1 and Stx2 affected kidney function, and although Stx2 inflicted more severe damage to the kidney than Stx1, the damage caused on the kidney by Stx1 was significant (Stearns-Kurosawa et al. 2013). In parallel, a differential susceptibility of endothelial cells to Stx1 and Stx2 has been described (Bauwens et al. 2011), but the real basis of these differences is not well understood. It has been suggested that holotoxin stability, enzymatic activity, and receptor affinity may be related to the differential toxicity of Stx1 and Stx2.

These toxins are characterized by an AB_5 structure with one enzymatically active A subunit non-covalently linked to a pentamer of B subunits responsible for the globotriaosylceramide (Gb3) binding. The Stx-Gb3 interaction leads to the internalization of the toxin which is transported via the retrograde pathway to the Golgi apparatus, endoplasmic reticulum, and nuclear membrane. An active A1 fragment

of the A subunit is retro-translocated into the cytoplasm where it binds to the large ribosomal subunit and inhibits protein synthesis by cleaving off a single adenine residue from the 28S rRNA. The action of Stx on target cells goes beyond the inhibition of protein synthesis. Further, it is well-known that the toxin also acts on cell signal transduction and immune modulation (Lee et al. 2013). The damage caused to the ribosome by Stx induces a ribotoxic stress response that is both pro-inflammatory and pro-apoptotic (Jandhyala et al. 2012). Indeed, the mechanisms of Stx1 and Stx2-induced apoptosis in various cell types have been extensively studied (Cherla et al. 2003). Recently, Basu and Tumer (2015) reviewed the participation of B subunits and the potential role for the A1 subunits in the differential toxicity of Stx1 and Stx2. Bentancor et al. (2013) studied the ability of the machinery of eukaryotic cells to recognize stx_2 sequences and to produce biologically active Stx2 in Vero cells transfected with a plasmid-encoding Stx2. Their results support the hypothesis that in the context of the inflammatory response of the colon during the EHEC (STEC) infection, phagocyte cells (macrophages/neutrophils) could incorporate stx_2 genes and produce active toxin as alternative source of Stx2.

The colon is the primary site of histological lesions caused by EHEC (STEC), and when the tissue is swollen, increases the Stx passage through the intestinal barrier, and the development of HUS is linked to the cytotoxic action exerted by Stx when passing from the intestine to the systemic circulation. Schüller (2011) reviewed the Stx interaction with the human intestinal epithelium and proposed that Stx uses different routes of translocation through the human intestinal epithelium in the early stages of infection: (1) Gb3-independent transcytosis, possibly enhanced by EHEC (STEC) infection; (2) paracellular transport during neutrophil (PMN) transmigration; (3) induction of Gb3 expression by EHEC (STEC) infection, retrograde transport, and Stx release after cell death; (4) Gb3-dependent translocation by Paneth cells; and (5) transcytosis by M cells. Sandvig et al. (2014) showed that the different steps of transport used by Stx have specific lipid requirements that confer specificity to Stx action.

Different studies have shown that kidney and brain are the most affected organs in HUS patients, due to the high sensitivity of the endothelial cells and renal microvasculature to Stx by the elevated expression of Gb3. Renal or neurological sequelae are consequences of irreversible tissue damage during the acute phase. Moreover, the tumor necrosis factor (TNF-α), mainly released by monocytes/macrophages, increases the expression of Gb3 in endothelial cells, and Stx is also able to increase and extend renal injury favoring endothelium interaction with leukocytes and platelets. Edema and detachment of endothelial cells of the basement membrane is seen in the histopathological examination of renal glomeruli and also formation of microthrombi of platelets rich in fibrin, causing the injury known as thrombotic microangiopathy (TMA). In addition to the direct effects of Stx on the renal epithelium and endothelium causing cell injury, the toxin induces an extensive inflammatory response and promotes the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), TNF-α, and IL-6 in human renal epithelial cells and increases the expression of chemokines, cytokines, and molecules of adhesion in epithelia and

endothelium, which contribute to renal injury observed in HUS. Stx2 also induces the increase of IL-8 and monocyte chemoattractant protein-1 (MCP-1) and fractalkine/CX3CL1 in human endothelial cells, promoting adhesion and leukocyte chemotaxis (Zoja et al. 2010).

Other virulence factors, such as lipopolysaccharide (LPS), are necessary for the full development of HUS. It is known that the joint action of Stx and LPS increases the production of nitric oxide (NO) and reduces levels of catalase. Gómez et al. (2013) have demonstrated that Stx induces an oxidative imbalance, evidenced by renal glutathione depletion and increased lipid membrane peroxidation. The enlarged production of reactive oxygen species by neutrophils could be one of the major sources of oxidative stress during Stx intoxication. The authors concluded that Stx generates a pro-oxidative state that contributes to kidney failure, and exogenous anti-oxidants could be beneficial to counteract this pathogenic pathway.

On the other hand, one should consider that the differences in virulence observed among EHEC (STEC) isolates may also be associated to a diversity of Stx expression. As the production of Stx relates to the level of phage production, considerable information about the characteristic and behavior of *stx* phages, the factors involved in their induction and dissemination, has contributed to our understanding on how phage variability may affect pathogenesis and disease (reviewed in Krüger and Lucchesi 2015). Moreover, the effect of some antibiotics on *stx* phage induction and Stx production presents adverse clinical consequences and directly impacts therapeutic approaches (McGannon et al. 2010). As such, studies on novel strategies trying to detect conditions that repress phage induction will certainly contribute to diminish the risk of development of HUS (Keen 2012).

The importance of Stx in the development of disease is unquestionable, but a diverse array of additional virulence factors, including adhesins, other toxins, and proteases, are involved in the establishment and maintenance of an infection.

The ability to colonize the host intestinal epithelium is considered a key step in pathogenesis. The presence of locus of enterocyte effacement (LEE), a chromosomal pathogenicity island (PAI), encoding a type III secretion system (TTSS), an outer membrane protein called intimin, and effector proteins responsible for the characteristic attaching-and-effacing (A/E) lesion is pathognomonic of disease related to EHEC (STEC). The A/E lesion is a histopathologic alteration characterized by the effacement of microvilli and the formation of pedestal-like structures in a process driven by the actin polymerization. The loss of the absorptive capacity of microvilli probably contributes to the diarrhea induced by these bacteria. Both O157:H7 (Wick et al. 2005) and non-O157 EHEC (STEC) strains (Karmali et al. 2003; Wickham et al. 2006) contain a variable repertoire of virulence determinants, including a collection of non-LEE encoded effector (*nle*) genes that encode translocated substrates of TTSS. The TTSS facilitates the persistence, host-to-host spread, and virulence of EHEC (STEC). However, although LEE has been linked to EHEC (STEC) strains belonging to major serotypes causing more than 80 % of HC and HUS cases in Europe (ECDC 2015a) and the United States (CDC 2013), it has been noticed that possession of LEE is not a mandatory condition for the occurrence of

exacerbated infections, as some LEE-negative strains are also capable of causing outbreaks and sporadic cases of HUS (Paton et al. 1999; Karch et al. 2005; Bielaszewska et al. 2011). Thereafter, an increasing number of adhesins composed by several fimbrial proteins, as well as different members of autotranporter proteins (AT) family, have been identified in EHEC (STEC) strains, some of them exclusively among LEE-negative strains, which were demonstrated to be involved in adherence to human epithelial cells as well as in biofilm formation (McWilliams and Torres 2014).

Besides Shiga toxins, the contribution of other toxins as the cytolethal distending toxin V (CDT-V) and subtilase cytotoxin (SubAB), produced by some particular serotypes, in the development and pathogenesis of HUS has been addressed (Bielaszewska et al. 2005; Paton and Paton 2010). Amaral et al. (2013) compared the effects of SubAB with those caused by Stx2 on primary cultures of human glomerular endothelial cells (HGEC) isolated from fragments of human pediatric renal cortex. Both toxins decrease the cell viability, but Stx2 caused a necrosis significantly higher than that induced by SubAB. Stx2 increased apoptosis in a time-dependent manner, while SubAB increased apoptosis at 4 and 6 h but decreased at 24 h. Pre-incubation of HGEC with C-9, a competitive inhibitor of Gb3 synthesis, protected HGEC from Stx2 but not from SubAB cytotoxic effects. These data provide evidence of how SubAB could cooperate with the development of endothelial damage characteristic of HUS pathogenesis.

In addition, a plasmid-encoded enterohemolysin (EhxA), which is a pore-forming cytolysin, has been identified at high frequencies among several EHEC (STEC) strains, and frequently associated with diarrheal disease and HUS. Despite EhxA contributing to disease by damaging the membrane of erythrocytes and other cells, its role in the hemolytic anemia of HUS patients has not been ascertained. Nevertheless, the enterohemolytic phenotype has been used as a good marker in the identification of EHEC (STEC) (Beutin et al. 1989).

The zoonotic character of EHEC (STEC) infections is well-established. Bacteria are largely distributed in the gastrointestinal tract of a wide diversity of animal species, normally as asymptomatic carriers. Several ruminant animals, especially cattle, are considered as the main natural reservoir, but other livestock species, domestic and wild mammals, birds, and fishes, have also been implicated in EHEC (STEC) carriage. The role of animals as reservoirs for infection or as spillover hosts has recently been reviewed (Persad and LeJeune 2014). As consequence, the transmission routes of EHEC (STEC) to human can occur either by the food chain, direct contact with animals or their environment, or by person-to-person spread. Infections have also been caused by drinking or swimming in contaminated water (Launders et al. 2013; Luna-Gierke et al. 2014). The panel of foods implicated as vehicles of EHEC (STEC) transmission is highly diverse. The main risk factors for EHEC (STEC)-associated human infections identified in earlier case-control and population-based studies were dietary behaviors and beef consumption. However, in recent years, other risky exposures have also emerged, like the consumption of fresh produce and sprouts, responsible for important outbreaks of HUS in several countries in the last 10 years (Beutin and Martin 2012; Rivas et al. 2014).

Therefore, several interventions targeting EHEC (STEC) related to animal handling, from farm to slaughter, as well as the implementation of food safety throughout production, processing, and distribution of fresh produce have been developed in past years looking for the improvement of the microbiological quality of foods.

The search for effective pre-harvest food safety practices for application to live cattle to reduce the contamination with *E. coli* O157 and other EHEC (STEC) strains of both foods of bovine origin, and related environmental contamination, has been reviewed by Besser et al. (2014). Different interventions, like feed ingredients, probiotics, and vaccines, have been identified with significant impact on *E. coli* O157 cattle shedding. But the authors concluded that the impact of these potential interventions remains insufficient due to their limited efficacy, practical difficulties with their implementation, and inconsistency in their results, leading to limited uptakes by producers. Also, the peri- and post-harvest interventions in the control of EHEC (STEC) in beef and in the agro-food chain were compiled by Moxley and Acuff (2014) and Duffy and McCabe (2014), respectively.

Since EHEC (STEC) does not cause disease in cattle, or triggers a local and protective immune response in the gastrointestinal tract, the goal of the EHEC (STEC) eradication from the bovine reservoir designing vaccines or other practices is not an easy task. Vaccines targeting a number of STEC O157-specific antigens have been tested in animal challenge studies. Some products have demonstrated efficacy to reduce the prevalence of cattle O157 shedding, but their efficacy to control the transmission in the environment during the natural exposure is doubtful (reviewed by Smith 2014).

Another strategy to decrease human-associated diseases involves the vaccination of the affected population, especially children. There are two possibilities: (1) a vaccine to control or prevent EHEC (STEC) infections; (2) a vaccine to prevent the systemic complications due to Stx action.

Because no licensed vaccine or effective therapy is presently available for human use, Mejias et al. (2014) recently developed a novel immunogen based on the B subunit of Stx2 and the enzyme lumazine synthase from *Brucella* spp. and they demonstrated the protection of mice against Stx2-associated damage by maternal immunization with the BLS-Stx2B chimera. Szu and Ahmed (2014) reviewed the human EHEC vaccines that have been studied clinically, in particular against *E. coli* O157. The LPS O157 conjugated to the recombinant exotoxin A of *Pseudomonas aeruginosa* (rEPA) has shown to be safe and induced high levels of anti-LPS, IgG antibodies, with bactericidal activity in adults and children (2–5 years old). On the other hand, a similar construct using the B subunit of Stx1 as the carrier protein elicited both bactericidal and toxin-neutralizing antibodies in mice.

There is a general agreement that patients with EHEC (STEC) infections should not be treated with antibiotics due to a higher risk of developing HUS as certain antibiotics induce expression of the Stxs. Recently, Agger et al. (2015) performed a systematic review in order to clarify the risk associated with the antibiotic treatment during acute EHEC (STEC) infections and in chronic carrier states. Among the ten clinical studies, four found an increased risk of HUS, four found no effect, and two found a reduced risk of HUS. In vitro and clinical studies suggested that DNA syn-

thesis inhibitors should be avoided, and certain protein and cell wall synthesis inhibitors reduced the toxin release from EHEC (STEC) isolates. The authors proposed that antibiotic treatment with protein and cell wall synthesis inhibitors could be considered when specific criteria (patient group, serotype, virulence profile, and duration of disease) are met. There are new therapeutic developments designed to limit Stx receptor generation or to prevent toxin binding, trafficking, processing, or activity within the cell (reviewed in Melton-Celsa and O'Brien 2014).

The identification of EHEC (STEC) strains of risk to public health is a challenge for diagnostic laboratories. A concept of molecular risk assessment (MRA) was developed by Karmali et al. (2003) and Coombes et al. (2008) as an aid to assess the role of genomic islands in contributing to the public health risk associated with different EHEC (STEC) strains, especially those found in foods, animals, or the environment. In this context, PCR-based methods could be used for identification of LEE-encoded effector and non-LEE-encoded effectors (*nle*) as an approach to define human virulent EHEC (STEC) types. Karmali et al. (2003) proposed a classification of STEC serotypes into five seropathotypes (A to E), taking into consideration the reported frequencies in human illness and associations with HC and HUS outbreaks. Seropathotype A (O157:H7 and O157:NM) is considered the most virulent and is related to the highest incidence in human disease and frequently involved in outbreaks. Seropathotype B (composed by O26:H11; O103:H2; O111:NM; O121:H19; and O145:NM) is associated at a lower frequency with severe human disease and uncommonly involved with outbreaks. Seropathotype C (O5:NM; O91:H21, O104:H21, O113:H21, and others) and D show a low incidence in human illness and are rarely associated with outbreaks, whereas seropathotype E is composed by many serotypes with no implication in human diseases so far demonstrated. Further, Coombes et al. (2008) identified 14 new *nle* genes in non-O157 STEC strains, grouped within three PAIs that correlated independently with outbreak and HUS potential for humans. Moreover, the authors showed an *nle* gene dosing effect in non-O157 STEC, where strains associated with severe human disease have an increased number of *nle* genes. Bugarel et al. (2010) have developed a low-density macroarray designed for simultaneous detection of genes encoding Stx_1 and Stx_2 (stx_1 and stx_2), intimin (*eae*), enterohemolysin (*ehxA*), and six different *nle* genes derived from genomic islands OI-71 (*nleF*, *nleH1-2*, and *nleA*) and OI-122 (*ent*, *nleB*, and *nleE*). The *nle* genes were found to be closely associated with certain serotypes and intimin genotypes in typical EHEC strains, including the new emerging EHEC (STEC) strains. The presence of *eae*, *ent*/*espL2*, *nleB*, *nleE*, and *nleH1-2* genes is a clear signature of EHEC (STEC) strains with high virulence for humans. Brandt et al. (2011) developed a PCR binary typing system (P-BIT) that could be used to aid in risk assessment and epidemiological studies of EHEC (STEC). They examined the distribution of 41 gene targets among O157 and non-O157 EHEC (STEC) isolates and found that P-BIT provided 100 % typeability for isolates, gave a diversity index of 97.33 % (compared with 99.28 % for *Xba*I pulsed-field gel electrophoresis [PFGE] typing), and produced 100 % discrimination for non-O157 STEC isolates. The authors identified 24 gene targets that conferred the same level

of discrimination and produced the same cluster dendrogram as the 41 gene targets initially examined. The P-BIT clustering identified O157 from non-O157 isolates and identified seropathotypes associated with outbreaks and severe disease. Using the MRA concept for screening EHEC (STEC) collections, an increasing number of emerging EHEC (STEC) types were detected (Bugarel et al. 2010). Internationally, the number of reported human diarrheal cases associated with non-O157 EHEC (STEC), including those leading to HUS, is rising rapidly, mainly due to increased surveillance for these pathogens.

However, the usefulness of the MRA concept changed dramatically in 2011. From May to July 2011, a large-scale outbreak was observed in several European countries, mainly affecting northern Germany, comprising 3842 cases of human infection, 855 (22.3 %) HUS cases and 53 fatalities, and involving an emerging enterohemorrhagic *E. coli* O104:H4 strain (Askar et al. 2011). This EHEC (STEC) strain presents an unusual virulence pattern that combines the production of Stx2a with enteroaggregative adherence, which is encoded by genes of the pAA plasmid and chromosomally carried genes of enteroaggregative *E. coli* (EAEC) strains (Bielaszewska et al. 2011). This new type of EHEC was designated enteroaggregative hemorrhagic *E. coli* (EAEHEC), since it shares virulence markers of both EAEC and EHEC strains. An important lesson to learn from the EAEHEC O104:H4 infection outbreak was that LEE and the *nle* genes, the current MRA approach to define human virulent EHEC (STEC) types, can be substituted by EAEC plasmid-encoded aggregative adherence mechanisms to enable Stx2-producing EAHEC to cause HC and HUS in humans (Beutin and Martin 2012).

After the 2011 outbreak and the fact that of all confirmed EHEC (STEC) infections in the European Union during 2007–2010, more than 85 % of the isolates were not fully serotyped, a Panel on Biological Hazards (BIOHAZ) was asked by the European Food Safety Authority (EFSA 2013) to review the seropathotype concept of Karmali et al. (2003) and the scientific criteria regarding pathogenicity assessment. The BIOHAZ Panel concluded that the seropathotype classification does not define pathogenic EHEC (STEC) nor does it provide an exhaustive list of pathogenic serotypes. The panel pointed out that there is no single or combination of marker(s) that defines a pathogenic STEC. Strains positive for stx_2 gene, and *eae* (intimin) or *aaiC* (secreted protein of EAEC) plus *aggR* (plasmid-encoded regulator) genes, are associated with a higher risk of more severe illness than other virulence gene combinations. Recently, de Boer et al. (2015) have described a rapid screening algorithm, including both molecular and conventional methods, to determine the pathogenic potential of STEC. The purpose was to discriminate infections with less-virulent EHEC (STEC) from those with clinical relevance and risk for public health.

During the investigation of the large outbreak reported in Germany in 2011, Whole Genome Sequencing (WGS) was applied for the first time in an epidemiology public health problem. The application involved the use of rapid, bench-top DNA sequencing technology, open-source data release, and prompt crowd-sourced analyses. In less than a week, these studies provided the draft genome of this new

EAEHEC hybrid and its unusual antibiotic resistance (Brzuszkiewicz et al. 2011). Afterwards, numerous advances in the use of WGS applied to research in different aspect of EHEC (STEC) were published (Eppinger et al. 2011; Jenkins et al. 2015).

A real-time evaluation of WGS for routine typing and surveillance of EHEC (STEC) was communicated by Joensen et al. (2014). The bioinformatics analysis using web-tools for species determination, multilocus sequence typing (MLST), determination of phylogenetic relationship, and a specific detection of *E. coli* virulence genes were described (Center for Genomic Epidemiology. http://www.genomicepidemiology.org). WGS demonstrated to be a robust method for assigning *stx* subtypes and for a real-time clustering of isolates in agreement with epidemiology, enabling discrimination between sporadic and outbreak isolates. Dallman et al. (2015) described the validation of the WGS approach as a molecular typing tool for surveillance of O157 in the United Kingdom and demonstrated that it can be used in real-time to provide the highest strain-level resolution for outbreak investigation.

There is an international agreement for the implementation of WGS for diagnosis and typing in Public Health due to the additional improvement in bioinformatics tools and the easy use in laboratories. However, WGS implies great changes. Regarding technology, new equipment would be needed for laboratories, and also for sequence analysis. Other major change is the way in which diagnostics and typing must be modified. It is recognized that the transition is a great challenge for the organizations and a concern to balance the benefits of applying new and powerful WGS approaches with the risk of implementing these new technologies too quickly (Joensen et al. 2014; ECDC 2015b).

2 Recent Advances in EHEC (STEC) Research

2.1 Emergence of New EHEC (STEC) Strains

The profile of the EHEC (STEC)-associated diseases has changed in recent decades, and different factors have contributed to the emergence of new strains causing sporadic cases and outbreaks with different epidemiological characteristics and widespread epidemics.

2.1.1 Emergence of a New EHEC (STEC) O26:H11 Clone in Europe

In Europe, EHEC (STEC) O26:H11 is the most common non-O157 serotype, being in some countries the most common cause of childhood-HUS. Previously, EHEC (STEC) O26:H11 strains isolated from humans harbored stx_{1a} and rarely associated with $stx_{2a.}$ However, a new highly virulent stx_{2a}-positive *E. coli* O26:H11 clone has been circulating in Europe since the mid-nineties (Bielaszewska et al. 2013). This clone was also observed in Latin America (Rivas et al. 2006b) and in the United

States (Brooks et al. 2005). By MLST analysis, the O26:H11 strains were divided into two phylogenetic groups: ST21 (stx_{1a} alone or associated with stx_{2a}), associated with less-severe disease, and ST29 (new stx_2 clone), with an increased virulence and disease severity. Recently, Delannoy et al. (2015) studied 23 *E. coli* O26:H11 strains, isolated from pediatric patients in France during the period 2010–2013. From the strains, 69.6 % belonged to the new clone, but 12 strains with negative results for both plasmid and chromosomal genetic markers exhibited a ST29 genotype and related CRISPR (clustered regularly interspaced short palindromic repeat) arrays, and seven strains harbored the stx_{2d} gene. By WGS, the evolutionary phylogenetic relationship of EHEC (STEC) O26:H11/H$^-$ has been investigated on European strains. Using the 48 phylogenetically informative single-nucleotide polymorphisms (SNPs), four distinct clonal complexes (CCs) were observed and the highly virulent German O26:H11 stx_{2a} clone was identified in a single CC, different from the former described strains (Bletz et al. 2013). Ison et al. (2016) differentiated *stx*-positive strains from *stx*-negative strains to infer the phylogenetic relationships of 178 *E. coli* O26:H11 bovine strains. The cattle *stx*-negative strains displayed synonymous SNP genotypes with the stx_2-positive, ST29 human strains, meanwhile the stx_1, ST21 human and cattle strains clustered separately, demonstrating the close phylogenetic relatedness of these *stx*-negative cattle strains and human clinical strains.

2.1.2 Emergence of EHEC/EAEC and Other Hybrid Strains Worldwide

Since the large outbreak associated with EAEHEC O104:H4 strain, attention has been focused on this new *E. coli* pathotype. Other O104:H4 strains with different PFGE macro-restriction profile, AAF type, antibiotic resistance, plasmid profile, virulence gene sets, or SNPs on the genomic level have been described, such as the HUSEC041 strain causing HUS in Germany in 2001, two French strains isolated in 2004 and 2009, and five additional strains causing HUS in France in 2011. As was previously proposed, it can be assumed that several O104:H4 EHEC lineages emerged from O104:H4 EAEC ancestors which differ in their genetic background (Mellmann et al. 2011). Like the enteroaggregative *E. coli* 55989 strain, isolated in Central Africa in the late 1990s, the German HUSEC041 isolate, the European outbreak strain, and the two French O104:H4 isolates belonged to ST678 and carried wzx_{O104}, $fliC_{H4}$, *aggR*, *lpfA*, *pic*, *sepA*, and *sigA*, and *stx-A/B2* genes (Monecke et al. 2011). The European outbreak strain harbored an extended-spectrum-lactamase gene, *bla*CTX-M-15, an additional lactamase gene (*bla*TEM-1), and other antibiotic resistance genes. Analysis of the genome sequences showed that HUSEC041 was positive for *bla*TEM-1, while the 2004 and 2009 French isolates lacked *bla*TEM-1 and other resistance genes. Ferdous et al. (2015) have described the isolation of *E. coli* O104:H4 strains in 2013, from a patient with HUS and a second individual showing only gastrointestinal complaints. They demonstrated that the EAEHEC O104:H4 Stx2a-positive strains were highly similar to the 2011 outbreak strain in

their core genome, showing that this clone is still circulating and a proper surveillance is necessary to prevent further outbreaks with these potentially pathogenic strains.

The emergence of EHEC/EAEC O104:H4 suggests that either certain EAEC serotypes might be more susceptible to acquire EHEC determinants or that there are certain EHEC/EAEC ancestors which successfully adapted to survival-specific selection conditions. In this context, other EHEC/EAEC strains of different serotypes were previously described associated with human disease. During an HUS outbreak in France in 1996, an O111:H2 strain was characterized as stx_2- and AAF-positive, and *eaeA*- and *ehxA*-negative, being the first EHEC/EAEC hybrid described (Morabito et al. 1998). Few years later, an O86:NM [H2] strain with stx_2 and AAF marker genes, but no *eae,* was isolated in 1999 from a pediatric patient with HUS and bloody diarrhea in Japan (Iyoda et al. 2000). In 2011, an O111:H21 strain associated with a household outbreak in Northern Ireland was detected. The strain harbored the stx_{2c} gene and the type V aggregative AAF fimbriae, but was *eae*-negative with a low level of resistance to ampicillin. It belonged to ST40, a sequence type comprising other *E. coli* pathotypes (STEC, EAEC, enteropathogenic *E. coli* [EPEC], and non-pathogenic *E. coli*). Genome sequencing revealed that the clonal complex, pAA plasmids, and phage encoded-*stx* genes were different in comparison with the O104:H4 outbreak strain (Dallman et al. 2012). Prager et al. (2014) screened about 2400 strains of the EHEC collection from the German National Reference Centre for *Salmonella* and other Bacterial Enteric Pathogens (NRC), corresponding to the period 2008–2012, for the presence of stx_1, stx_2, *eae,* and *ehxA* genes. Among 268 *eae*-and *ehxA*-negative strains, two strains exhibited both EHEC and EAEC marker genes and were stx_2- and *aatA*-positive. One strain, isolated from a bloody diarrhea patient in 2010 and serotyped as O59:H-[$fliC_{H19}$], harbored stx_{2a}, belonged to ST1136, and exhibited genes for type IV aggregative AAF fimbriae, and with resistance towards sulfonamides, streptomycin, and trimethoprim/sulfonamide. The *iha*, $lpfA_{O26}$, $lpfA_{O113}$, and *irp2* genes, frequently associated with EHEC, and *aggR*, *aap*, *set1a*, *set1b*, *pic*, *sigA,* and *iucA* genes in general related to EAEC were detected. The *astA* gene was not detected. The second strain isolated from a patient with diarrhea in 2012, harbored stx_{2b}, was typed as Orough:H2 and belonged to ST26. In Argentina, Carbonari et al. (2015) screened a total of 36 stx_2-positive, *eae*- and *ehxA*-negative non-O157 STEC strains, isolated from HUS and diarrhea cases, for the AraC-like regulator AggR. Nine (25 %) EHEC/EAEC O59:H-[$fliC_{H19}$] strains were identified, isolated from 8 HUS and one bloody diarrhea cases. The first O59 isolate corresponded to a HUS case from 2005. The stx_{2a}, *iha*, lpf_{O26}, lpf_{O113}, *aatA*, *aap*, *sigA* genes were detected. The presence of type IV aggregative AAF fimbriae was established by the amplification of the *agg4A* fimbrial subunit gene. The strains showed low toxicity on Vero cells and were resistant to streptomycin and trimethoprim/sulfonamides. By *Xba*I-PFGE, nine patterns were established, with 86.7 % similarity. A high clonal relationship (>85 %) with the EHEC/EAEC O59:H-[$fliC_{H19}$] German strain was established. Tozzoli et al. (2015) reported an outbreak caused by an EHEC/EAEC O127:H4 strain in Northern Italy in 2013. The analysis of 76 fecal samples from children and school staff was performed. Five Stx2-producing EAEC

O127 strains were isolated. By WGS, the strain was characterized as O127:H4 and ST678. It possessed the stx_{2a} gene and other EAEC virulence genes such as *aggR*, *aap*, *aat*, *aaiC*, *sigA*, *pic,* and *astA*. The stx_2-phage was inserted in *wrbA* site and was highly similar to that of the O104:H4 outbreak strain. Nyholm et al. (2015) have sequenced the whole genome of three (2 humans and 1 bovine) EHEC (STEC)/ETEC strains harboring both *stx* and *est* genes. They concluded that virulence genes of different *E. coli* pathogroups can coexist in strains of different phylogenetic lineages and the finding of novel hybrids is a challenge for the traditional diagnostic of *E. coli* infections.

2.1.3 Emergence of Hypervirulent EHEC (STEC) O157 Strains of Clade 8

Manning et al. (2008) showed that both outbreaks, in the United States in 1993 and Japan in 1996, had low rates of hospitalization and HUS in comparison with the 2006 North American spinach outbreak. Phylogenetic analysis identified 39 SNP genotypes in a broad collection of STEC O157 and allowed to separate the isolates into nine distinct clades. Patients with HUS were significantly more likely to be infected with strains of clade 8, which have increased in frequency over the past 5 years. It has been suggested that enhanced severity related to clade 8 strains may be explained by the overexpression of some genes, particularly stx_2 (Neupane et al. 2011). Moreover, Kulasekara et al. (2009) examined the genome of TW14359, the strain associated with the spinach outbreak in the United States, and they compared it to the genome of two other sequenced prototype strains (EDL933 and Sakai). They found seven coding sequences postulated as putative virulence factors that could be responsible for the high virulence of this strain. Lineage-specific polymorphic assay (LSPA-6), derived from octamer-based genome scanning by selecting six loci that are biased in their allelic distribution among *E. coli* O157 strains (Yang et al. 2004), allows the description of lineages I, II, and I/II that have a different distribution among different hosts and different geographical regions (Yang et al. 2004; Manning et al. 2008; Hirai et al. 2014; Mellor et al. 2015). Also, several studies have shown a marked difference on lineages distribution between human isolates and strains from the cattle reservoir (Whitworth et al. 2010; Franz et al. 2012). LI/II predominates in both clinical and bovine isolates in Argentina and Australia (Mellor et al. 2012). Pianciola et al. (2014) have described the almost exclusive circulation of *E. coli* O157 strains belonging to the hypervirulent clade 8 (>90 %) and also the presence of putative virulence factors in higher frequencies than those reported in Neuquén Province, Argentina, a region with one of the highest HUS incidence worldwide. Recently, the same group has demonstrated a high prevalence of EHEC (STEC) O157 clade 8 in human strains with the exclusive presence of LSPA-6 lineage I/II in strains from other regions of Argentina. This particular scenario may be originated in a similar situation in the bovine reservoir, with only slight differences. This homogeneity in EHEC (STEC) O157 genotypes detected in human and bovine strains contrasts with results reported in other countries (Pianciola et al. 2016).

2.2 Advances in the Knowledge of LEE-Negative EHEC (STEC) Strains Associated with Human Disease

EHEC (STEC) O113:H21 is a LEE-negative strain prevalent in the environment, which has been isolated from foods and animals and also from patients with severe disease. Feng et al. (2014) have described a PCR microarray and *stx* subtyping PCR to characterize 65 strains isolated from various sources (environment, food, and clinical infections) and geographical locations (Argentina, Brazil, Canada, and the United States, among others). All the strains carried only Stx subtypes associated with human infections, suggesting that the environmental strains have the potential to cause disease. Most of the O113:H21 strains were closely related and belonged to the same ST223 clonal group, but CRISPR analysis showed a great degree of genetic diversity among the O113:H21 strains. In recent years, another LEE-negative EHEC (STEC) strain serogrouped as O178 have been isolated from cattle and food of bovine origin in South America and Europe. Miko et al. (2014) characterized 74 German and Argentinean *E. coli* O178 strains from animals, food, and humans and studied their serotypes, *stx*-genotypes, and 43 virulence-associated markers by a real-time PCR-microarray. Most ($n=66$) of the strains belonged to serotype O178:H19 and were mainly isolated from cattle and food of bovine origin, but one strain was isolated from an Argentinean patient with HUS. Genotyping the STEC O178:H19 strains by PFGE revealed two major clusters: Cluster A-strains ($n=35$), including the HUS-strain, carried genes associated with severe disease in humans (stx_{2a}, stx_{2d}, *ehxA*, *saa*, sub_{AB1}, $lpfA_{O113}$, *terE* combined with stx_{1a}, *espP*, *iha*), and cluster B-strains ($n=26$) showed a limited repertoire of virulence genes (stx_{2c}, *pagC*, $lpfA_{O113}$, *espP*, *iha*). Based on these results, the authors recommended that EHEC (STEC) O178:H19 strains should be considered with respect to their potential to cause diseases in humans.

2.3 EHEC (STEC) in the Environment

Lascowski et al. (2013) reported the frequency and characteristics of EHEC (STEC) in 1850 treated and untreated drinking water samples, collected in 41 municipalities in the north of Paraná State, Brazil, in the period February 2005–January 2006. A total of 12 isolates, 11 from untreated water and one from treated water, were positive for stx_1/stx_2 (5), stx_1 (2), and stx_2 (5). All stx_2-positive isolates presented the $stx_{2dactivatable}$ subtype, were *eae*-negative, but carried other virulence genes such as *ehxA* (100 %), *saa* (100 %), $lpfA_{O113}$ (75 %), *iha* (42 %), sub_{AB} (25 %), and *cdtV* (8 %). Multidrug resistance was identified in 25 % of the isolates. The strains belonged to seven distinct serotypes and PFGE revealed the presence of two clusters and two clones in the region. The authors concluded that the analysis of the drinking water supplies for pathogenic *E. coli*, as EHEC (STEC), may be useful to prevent waterborne outbreaks. Tanaro et al. (2014) reported the isolation of *E. coli* O157:H7 from

311 surface water samples exposed and not exposed to runoff from corrals, near 48 cattle feedlots, distributed in the Province of Entre Ríos, Argentina, in the period April 2009–July 2011. By multiplex PCR, 70.5 % of the exposed surface water (ESW) samples were rfb_{O157}-positive, and 62 *E. coli* O157 and 32 EHEC (STEC) O157:H7 strains were isolated. In the non-exposed surface water (NESW) samples, 60.0 % were rfb_{O157}-positive, 9 *E. coli* O157, and 6 EHEC (STEC) O157:H7 strains. Although no significant differences were found, these results showed that the ESW tended to be more contaminated with EHEC (STEC) O157:H7 than NESW.

These findings highlight the relevance of the persistence of EHEC (STEC) in the environment as a result of the extensive livestock farming, and the risk that pathogens contained in feedlot runoff may reach recreational waters and also contaminate produce through irrigation, increasing the potential dissemination of O157 strain and the subsequent risk for humans. Matheus-Guimarães et al. (2014) have determined the ability of 14 O157 and 8 non-O157 strains, isolated from bovine hide and carcass, to interact with biotic and abiotic surfaces. Biofilm formation assays showed that four O157 and two non-O157 strains were able to adhere to glass, and only one O157 strain to polystyrene. The data suggested that STEC strains can have different factors involved in the biofilm production on diverse surfaces. The ability of non-O157 LEE-negative strains to form biofilm highlights an industrial and health problem that cannot be ignored. Moreover, the detection of an O157 EHEC (STEC) strain that is able to form biofilm on different surfaces and adhere to and invade human cells indicates an important ability to persist in the environment and to interact with the host.

3 EHEC (STEC) in Latin America

3.1 Surveillance of EHEC (STEC) Infections Tends to Integrate Food Chain Surveillance Systems

In general, there are different types of food-borne surveillance systems, including event-based surveillance, indicator-based surveillance, and integrated food chain surveillance. Each country must determine the most appropriate structure for their surveillance system based on their available resources. In America, there are different implemented surveillance systems, depending on the country. The industrialized countries show integrated food chain surveillance systems established according to national regulations and including different networks that work together with standardized protocols. Integrated food chain surveillance is viewed as the optimal practice for conducting continuous risk analysis for food-borne diseases, but also requires significant ongoing resources and greater multidisciplinary collaboration compared to the other systems (Ford et al. 2015).

Different authors have described the surveillance implemented in the United States and Canada, demonstrating how the surveillance system has the capability to

help assess the magnitude of the food safety problem, define priorities for action, establish transmission pathways and food sources, provide different control options, define targets along the food chain, and measure the success of food safety interventions (Havelaar et al. 2007; Gaulin et al. 2014; Whitney et al. 2015).

In the United States, *E. coli* O157:H7 infections became nationally notifiable in 1995. Since 2000, all EHEC (STEC) infections that cause human illness are notifiable to the Nationally Notifiable Diseases Surveillance System (NNDSS). The Foodborne Diseases Active Surveillance Network (FoodNet) monitors the incidence of laboratory-confirmed infections caused by nine pathogens commonly transmitted through food, including O157 and non-O157. In 2014, 690 cases of non-O157 EHEC (STEC) and 445 cases of O157 were notified, with incidence rates of 1.43 and 0.92/100,000 population, respectively. Among 546 (79%) serogrouped non-O157 isolates, the top O-groups were O26 (31%), O103 (24%), and O111 (19%). Compared with the 2011–2013 period, the incidence of EHEC (STEC) O157 infections was lower, while the incidence of non-O157 infections increased. In 2013, a total of 87 cases of post-diarrheal HUS were reported among children aged <18 years (0.79 cases per 100,000). Of these, 46 (53%) occurred in children aged <5 years (1.55 cases per 100,000) (Crim et al. 2015).

In Canada, EHEC (STEC) infection has been classified as a notifiable disease since 1990. FoodNet Canada's (formerly known as C-Enter Net) is the comprehensive and integrated surveillance system that focuses on active surveillance of human cases of illness, coupled with monitoring possible sources of illness in food, animals, and water. In 2013, 470 *E. coli* O157 cases occurred (1.34 cases/100,000 populations), with 245 hospitalizations and 8 fatalities. For each *E. coli* O157 case reported to Canada's National Surveillance System, it is estimated that there are approximately 20 cases in the community (Government of Canada 2015).

3.2 Surveillance and Epidemiology of EHEC (STEC) Infectious Diseases in Latin America

In Latin America, the EHEC (STEC) surveillance systems are different in each country, and they were implemented according to priorities in public health and resources. In recent years, the countries have enhanced their strategies, working in agreement with different partners (other countries, PAHO, WHO, CDC, PulseNet, Sanger Institute). The need to respond to different epidemiological situations at national, regional, and international levels motivated countries to get an improvement in diagnosis and subtyping. Furthermore, since national surveillance systems for EHEC (STEC) have improved, an increased report in number of clinical cases and etiologic agent detection was observed.

The report of EHEC (STEC) infectious diseases and HUS cases is different in each country. In general, the report relies primarily on syndromic surveillance through the food-borne diseases surveillance System (Argentina, Bolivia, and

Paraguay) and/or through the acute diarrheal surveillance system (Argentina, Brazil, Chile, Costa Rica, Paraguay, and Peru). The official notification of HUS is mandatory in Argentina, Bolivia, Brazil, Chile, and Paraguay. Countries like Uruguay and Costa Rica do not have a formal surveillance system for HUS and EHEC (STEC) infections. In general, the syndromic surveillance is reinforced with laboratory-based surveillance through their National Networks. Depending on the laboratory capacity, resources, and infrastructure, molecular methods for EHEC (STEC) detection were implemented at different levels, in each country. Mostly, the preliminary results in local laboratories are still generated by isolation and phenotypic methods (serotyping, enterohemolysin detection, among others) and then strains are submitted to the National Reference Laboratory (NRL) for further confirmation (by PCR, MLVA, PFGE). The NRLs participate in the Network's External Quality Assurance System (EQAS; National Food Institute, Denmark / Global Foodborne Infections Network) for quality assurance evaluation for food-borne pathogens diagnosis and/or more specifically in the Quality Assurance for EHEC (STEC) diagnosis and subtyping (Staten Serum Institute, Denmark). Additionally, countries are evaluated for EHEC (STEC) subtyping by PFGE through the PulseNet Latin America and the Caribbean (PNALC) QAQC program (certification and proficiency testing) established according to the requirements of PulseNet International (PNInt).

In Argentina, post-diarrheal HUS is endemic and the prevalence is the highest worldwide. Data on human EHEC (STEC) infections are gathered through different strategies: (1) the National Health Surveillance System collects data of HUS cases, and since 2000, the report is mandatory and must be immediate and individualized; (2) the Sentinel Surveillance System through 25 HUS Sentinel Units; (3) the Laboratory-based Surveillance System through the National Diarrheal and Foodborne Pathogens Network; and (4) the Molecular Surveillance through the PNALC. Over the last 10 years, around 400 HUS cases were reported annually. In the period of 2010–2015, the median of incidence was 8.4 cases per 100,000 children <5 year of age and the lethality was between 2 and 5 %. Most (36 %) of the cases were children <5 years old and 56 % were female (Ministerio de Salud 2016. http://www.msal.gov.ar/index.php/home/boletin-integrado-de-vigilancia). In 2015, 337 HUS cases were notified and 190/257 (73.9 %) EHEC (STEC) infections were confirmed at LNR. O157:H7 (56.3 %) was the predominant serotype, with the stx_{2a}/stx_{2c}/*eae*/*ehxA* genotype, followed by O145:H-[$fliC_{H28}$] (13.4 %), stx_{2a}/*eae*/*ehxA*. Recently, an indirect diagnostic of antibodies against EHEC (STEC) O157, O145, and O121 by ELISA, using glycoconjugates (glyco-iELISAs), was implemented, representing an improvement in the diagnostic, especially in those cases where the isolation was not possible (Melli et al. 2015).

In general, HUS cases are sporadic; however, some outbreaks are reported through the surveillance system of HUS and associated diseases. In the period 2013–2015, 27 outbreaks of bloody diarrhea and HUS, associated with O157 and non-O157 STEC strains (24 in families, 2 in kindergartens, and 1 in the community), were identified. The first detection of hybrid strains (Carbonari et al. 2015) was a laboratory finding, and the NRL was forced to modify the screening workflow for a broader molecular characterization to enhance the sensitivity in the diagnosis.

In Chile, the clinical surveillance of HUS and laboratory-based surveillance for EHEC (STEC) infections are mandatory at national level and establish to send the isolates to the Instituto de Salud Pública as NRL for further confirmation. In the period 2007–2013, 599/2425 (24.7 %) of the strains received were confirmed as EHEC (STEC). The predominant serotypes were O157:H7 (52.7 %), O26:H11 (24.9 %), and O26:H− (7.3 %). The majority of cases were from the Región Metropolitana (74.0 %), 52.2 % were males and the most affected group corresponded to children aged 1–4 years old (Ministerio de Salud 2014. http://www.ispch.cl/sites/default/files/STEC.pdf).

In Uruguay, the report of HUS cases is included in food-borne diseases outbreaks or public health events of national significance and become immediate notification. The incidence of HUS is approximately 5/100,000 children <5 years old, with 12–15 new cases annually. Among 43 children with clinical diagnosis of post-diarrheal HUS studied (2002–2013), in seven cases EHEC (STEC) strains of different serotypes and genotypic profiles (O157:H7, *stx*$_2$/*eae*$_{\gamma1}$/*ehxA*, O26:H11, *stx*$_1$/*eae*$_{\beta1}$/*ehxA*, O26:H−, *stx*$_1$/*stx*$_2$/*eae*$_{\beta1}$/*ehxA*, O111:H−, *stx*$_1$/*stx*$_2$/*eae*$_{\gamma2}$/*ehxA*, O145:HNT, *stx*$_2$/*eae*$_{\beta2}$/*ehxA*, and ONT, *stx*$_2$/*eae*/*ehx*A) were recovered. In one case, a co-infection (O26:H11/O145:HNT) was detected (Varela and Schelotto 2015). In 2012, extra-intestinal O157:H7 infections in two elderly women were reported for the first time in Uruguay. The strains were characterized as *stx*$_1$/*eae*$_{\gamma1}$/*ehxA*/*fliC*$_{H7}$/*fimA* of phage type (PT) 39 and *stx*$_1$/*stx*$_2$/*eae*$_{\gamma1}$/*fliC*$_{H7}$/*fimA* of PT40 (Gadea et al. 2012). In 2010–2011, a descriptive study was conducted to determine etiology and clinical manifestations of acute diarrhea in children up to 5 years of age from high socioeconomic level households. Out of 59 diarrheal cases, two O26 and one O153 EHEC (STEC) strains were detected. A child infected with EHEC (STEC) O26 *stx*$_1$/*stx*$_2$/*eae*$_{\beta1}$/*ehxA* strain, who had bloody diarrhea, developed a complete HUS after 20 days, requiring dialysis in the acute stage (Varela and Schelotto 2015).

In Brazil, EHEC (STEC) infections are important public health issues in some regions, but in general the incidence is relatively low (Guth et al. 2010). The HUS surveillance is mandatory at national level and the EHEC (STEC) surveillance is performed through monitoring diarrheal diseases, targeting mainly the detection of diarrhea outbreaks. Furthermore, each state could have the own regulations with specific programs to reinforce the national surveillance. The HUS Laboratory Network consists of five Sentinel Laboratories and the Instituto Adolfo Lutz is the NRL, to which all strains are submitted for further characterization and subtyping. Human infections are linked mostly to sporadic cases of non-bloody diarrhea associated mainly with non-O157 strains. However, HUS cases associated with O157 as well as non-O157 infections have been described in São Paulo State. Almost half (46 %) of patients were <2 years old and female (61.5 %). EHEC (STEC) strains were isolated from 3/7 patients, and serotypes O26:H11 (*stx*$_1$/*eae*/*ehxA*), O157:H7 (*stx*$_{2a}$/*stx*$_{2c}$/*eae*/*ehxA*), and O165:HNM (*stx*$_{2a}$/*stx*$_{2c}$/*eae*/*ehxA*) were identified (de Souza et al. 2011). The results of the indirect diagnosis by LPS antibodies-ELISA showed that seven sera yielded positive signal for O157 LPS-antibodies and 2 for O111 LPS-antibodies (de Souza et al. 2011). In the Rio de Janeiro State, from a total

of 1154 strains received in the period 2013–2015 by NRL for Enteric Diseases at the Instituto Oswaldo Cruz (FIOCRUZ), 42 (3.6 %) were confirmed as non-O157 EHEC (STEC). The origin of the strains was human (24), foods (2), environment (1), and animal (15) (Rodriguez, personal communication).

In Bolivia, the surveillance has been improved through a South-South Cooperation Project with Argentina. In 2/3 HUS cases notified in 2014–2015, O157/stx_2 and O26/stx_1 strains were isolated from children <2 years old. Also, 30 EHEC (STEC) O157 and non-O157 strains were detected in ground beef during sampling procedures at retail stores, conducted in 2010–2013 by the Red de Laboratorios Oficiales de Análisis de Alimentos (RELOAA), in La Paz, Cochabamba, Tarija, Sucre and Santa Cruz Departments. The highest detection rate (56.6 %) was in La Paz and El Alto. All O157:H7 strains ($n = 9$) were stx_{1a}/stx_{2a}/*eae*/*ehxA*, except one that was stx_{2a}. The non-O157 isolates were O8:H19 stx_{2b}/*saa*/*ehxA*, O91:HNT stx_{2b}/*saa*/*ehxA*, O126:H27 stx_1/*saa,* and ONT:H28 stx_1/*saa*/*ehxA* (1, each one), while 18 strains were not serotyped. (Damiani and Montiveros, personal communication).

In Paraguay, the HUS notification is mandatory, immediate, and individualized. The EHEC (STEC) infections are gathered by the laboratorial surveillance of diarrheal and food-borne diseases (Ministerio de Salud y Bienestar Social, 2015. http://vigisalud.gov.py/wp-content/uploads/2015/12/GNVNPY.pdf). In 2013–2015, ten HUS cases without EHEC (STEC) isolation were notified. However, strains were detected in eight sporadic diarrhea cases and characterized as O26 stx_1/stx_2/*eae*, ONT:HNT stx_1/*eae*, ONT:HNT stx_2, and ONT:HNT stx_1/stx_2, ONT:HNT stx_1 (Weiler, personal communication).

In Costa Rica, EHEC (STEC) infections are not notifiable in the surveillance system. However, the Centro Nacional de Referencia de Bacteriología (CNRB) of Inciensa has implemented a differential diagnostic protocol for pathogens associated to diarrheal cases, mostly involved in outbreak or dead. Through this strategy in 2013–2015, 11 EHEC (STEC) strains O157:H7/stx_2/*eae* (2), O145/stx_2/*eae* (3), non-O157 stx_1/*eae* (4), and non-O157 *stx*1 (2) were isolated from sporadic childhood diarrheal cases. The CNRB also receive for confirmation purposes strains isolated from water and food from the Laboratory Network and from animals submitted by the Laboratorio Nacional de Servicios Veterinarios (LANASEVE) depending on the Servicio Nacional de Sanidad Animal (SENASA, Ministerio de Agricultura). Throughout sampling procedures in four exporting food plants, performed by the Dirección de Inocuidad de Productos de Origen Animal (DIPOA), using the USDA/FSIS guidelines, 18 non-toxigenic O157:H7, three O157 stx_1/stx_2, one O157 stx_2, and one non-O157 stx_1 strains were detected (Bolaños and Duarte, personal communication).

In Peru, the surveillance of EHEC (STEC) infections is performed through the monitoring of acute diarrheal diseases. The Instituto Nacional de Salud as NRL has implemented molecular protocols for laboratorial-based surveillance. Four O157:H7 strains genotyped as stx_2 (2), stx_1, and stx_1/stx_2 were isolated from diarrheal cases attended in Lima in 2014. One HUS case was reported in Lambayeque without EHEC (STEC) isolation (Zamudio, personal communication).

3.3 Technologic Improvement of Latin America Laboratories for the Integration of the Region in the Worldwide EHEC (STEC) Surveillance

Laboratory-based surveillance for detection of EHEC (STEC) is a key in the surveillance of associated diseases, globally. Actually, the international trends indicate that just the improvement of the national surveillance is not enough and it is essential to get a worldwide coverage. Collaboration and data sharing between organizations and countries is required due to the international dimension of food-borne pathogens and food trade in particular.

Reference Laboratories of Central and South America participate regionally in the Global Foodborne Infections Network (GFN) that give support in diagnosis capability and improve the response capacity of food-borne diseases. As part of PNLAC, NRLs contribute to the molecular surveillance of food-borne pathogens, in the framework of PNInt.

In the PNALC Regional Database, there are 498 PFGE patterns, corresponding to 985 STEC O157 strains isolated in 1988–2015 in five countries (Argentina, Chile, Cuba, Paraguay, and Uruguay). A high genetic diversity among the strains of different countries (63.1 %) is observed, and certain PFGE patterns highly related are detected in specific countries. Interestingly, there are strains with identical patterns circulating in Argentina and Chile. PNALC offers to the countries the possibility to join efforts, working with standardized protocols under a quality control system that let to participate actively in the worldwide surveillance. The network is continually working trying to get harmonization between countries in the implementation and performance of novel technologies. At present, the vision of PNInt is the worldwide use of WGS in all public health laboratories to identify, characterize, and subtype foodborne bacterial pathogens, replacing existing phenotypic and molecular methods as support of food-borne disease surveillance and thus reach the reduction of the burden of these diseases. This proposal includes mainly the use of wg-MLST as strategy for sequence analysis. Because of the public health risk, the first approach on wg-MLST was a pilot project on *Listeria* and now continues with EHEC (STEC). For this strategy, it is needed to build an allele database stored in a unique engine server. Standardized protocols, validation, and nomenclature designation are in progress in order to work on the harmonization of the strategies among the countries globally.

All members of PNInt, including PNLAC, agree to transition to WGS. The situation in countries of Latin America could be briefly described in three items, (1) the wide range of capability. The first steps in training were done by courses/workshops in the framework of PNALC in collaboration with the Wellcome Trust Sanger Institute. Moreover, some countries are in the stage of equipment acquisition or installation (Paraguay and Venezuela), and others have already implemented WGS, but the current use is just for research, no for routine surveillance (Argentina, Chile, Colombia, México, and Peru); (2) the availability of resources to WGS is variable among countries and maybe it is the major weakness to overcome; (3) some barriers, like bioinformatics capacity for analysis and storage, and connectivity issues should be improved.

At present, Argentina is participating as external laboratory, in a Pilot Project of WHO/FDA for WGS implementation to support public health surveillance, in the framework of the Genome TRAKR Project. Because of the endemic situation of HUS and EHEC (STEC) infectious diseases in the country, a specific project was included. In order to determine the concordance between routine tests and WGS results regarding detection and characterization issues for diagnosis and discrimination and relationships among strains for outbreak and clade detection, the LNR has run 16 EHEC (STEC) O157:H7 strains of different clades, sources, and different date of isolation. The obtained sequences had comparable coverage and genome size and passed QC assessment, and they were accessible at NCBI (BioProject PRJNA282762). An agreement with previous results, mainly regarding identification, characterization, outbreak, and clade detection, was observed. By WGS, additional information like other virulence factors, ST and phylogenetic tree, could be analyzed (Chinen et al. 2015).

4 Conclusions

Advances on the knowledge of pathogenesis, virulence determinants, and risk factors have contributed to the development of several strategies trying to prevent food and environment contamination, and consequently transmission to humans. The uses of new techniques, like WGS typing, has been useful in surveillance, diagnosis, and epidemiological studies, as well as the discovery of emerging genotypes and identifying the genetic differences between human pathogenic and nonpathogenic EHEC (STEC) strains.

Acknowledgment The data reported here is a summary of the efforts of many individuals and working groups in Latin America. Special thanks to E. Damiani, D. Montiveros (INLASA, Bolivia); V. Dias Gonçalves, M. Lima Festivo, D. Rodriguez (Instituto Oswaldo Cruz, Brazil); LF dos Santos, C. Camargo (Instituto Adolfo Lutz, Brazil); H. Bolaños, F. Duarte (INCIENSA, Costa Rica); S. Ureña (COOPESALUD, Costa Rica); V. Soto (SENASA, Costa Rica); N. Weiler Gustafson, V. Orrego (INS, Paraguay); ML. Zamudio (INS, Perú); F. Schelotto, G. Varela (Instituto de Higiene, Uruguay).

References

Agger M, Scheutz F, Villumsen S et al (2015) Antibiotic treatment of verocytotoxin-producing *Escherichia coli* (VTEC) infection: a systematic review and a proposal. J Antimicrob Chemother 70:2440–2446

Amaral MM, Sacerdoti F, Jancic C et al (2013) Action of Shiga toxin type-2 and subtilase cytotoxin on human microvascular endothelial cells. PLoS One 8(7), e70431

Ardissino G, Possenti I, Tel F et al (2014) Time to change the definition of hemolytic uremic syndrome. Eur J Intern Med 25(2), e29

Askar M, Faber MS, Frank C et al (2011) Update on the ongoing outbreak of haemolytic uraemic syndrome due to Shiga toxin-producing *Escherichia coli* (STEC) serotype O104, Germany, May 2011. Euro Surveill 16(22). pii: 19883. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19883

Basu D, Tumer NE (2015) Do the A subunits contribute to the differences in the toxicity of Shiga toxin 1 and Shiga toxin 2? Toxins 7:1467–1485

Bauwens A, Bielaszewska M, Kemper B et al (2011) Differential cytotoxic actions of Shiga toxin 1 and Shiga toxin 2 on microvascular and macrovascular endothelial cells. Thromb Haemost 105:515–528

Bentancor LV, Bilen MF, Mejías MP et al (2013) Functional capacity of Shiga-toxin promoter sequences in eukaryotic cells. PLoS One 8(2), e57128

Besser T, Schmidt C, Shah D et al (2014) "Preharvest" food safety for *Escherichia coli* O157 and other pathogenic Shiga toxin-producing strains. Microbiol Spectr 2(5):EHEC-0021-2013

Beutin L, Martin A (2012) Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot 75:408–418

Beutin L, Montenegro MA, Ørskov I et al (1989) Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. J Clin Microbiol 27:2559–2564

Bielaszewska M, Sinha B, Kuczius T et al (2005) Cytolethal distending toxin from Shiga toxin-producing *Escherichia coli* O157 causes irreversible G2/M arrest, inhibition of proliferation and death of human endothelial cells. Infect Immun 73:552–562

Bielaszewska M, Mellmann A, Zhang W et al (2011) Characterization of the *E. coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect Dis 11:671–676

Bielaszewska M, Mellmann A, Bletz S et al (2013) Enterohemorrhagic *Escherichia coli* O26:H11/H-: a new virulent clone emerges in Europe. Clin Infect Dis 56:1373–1381

Bletz S, Bielaszewska M, Leopold SR et al (2013) Evolution of enterohemorrhagic *Escherichia coli* O26 based on single-nucleotide polymorphisms. Genome Biol Evol 5:1807–1816

Brandt SM, King N, Cornelius AJ et al (2011) Molecular risk assessment and epidemiological typing of Shiga toxin-producing *Escherichia coli* by using a novel PCR binary typing system. Appl Environ Microbiol 77:2458–2470

Brooks JT, Sowers EG, Wells JG et al (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. J Infect Dis 192:1422–1429

Brzuszkiewicz E, Thürmer A, Schuldes J et al (2011) Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: entero-aggregative-haemorrhagic *Escherichia coli* (EAHEC). Arch Microbiol 193:883–891

Bugarel M, Beutin L, Fach P (2010) Low-density macroarray targeting non-locus of enterocyte effacement effectors (*nle* genes) and major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC): a new approach for molecular risk assessment of STEC isolates. Appl Environ Microbiol 76:203–211

Carbonari C, Miliwebsky E, Deza N et al (2015) Novel EAEC/STEC hybrid O59:NM[H19] strains isolated from human infections in Argentina. In: Abstracts of the ninth triennial international symposium on Shiga toxin-producing *Escherichia coli* infections, Boston, 13–16 Sept 2015

Centers for Disease Control and Prevention. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet (2013) annual report. http://www.cdc.gov/foodnet/reports/annual-reports-2013. Accessed 22 Feb 2016

Cherla RP, Lee SY, Tesh VL (2003) Shiga toxins and apoptosis. FEMS Micobiol Lett 228:159–166

Chinen I, Carbonari C, Campos J et al (2015) Implementation of whole genome sequencing based STEC surveillance in Argentina. In: Abstracts of the ninth triennial international symposium on Shiga toxin-producing *Escherichia coli* infections, Boston, 13–16 Sept 2015

Coombes BK, Wickham ME, Mascarenhas M et al (2008) Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing *Escherichia coli* strains. Appl Environ Microbiol 74:2153–2160

Crim SM, Griffin PM, Tauxe R et al (2015) Preliminary incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2014. MMWR Morb Mortal Wkly Rep 64:495–499

Dallman T, Smith GP, O'Brien B et al (2012) Characterization of a Verocytotoxin-producing enteroaggregative *Escherichia coli* serogroup O111:H21 strain associated with a household outbreak in Northern Ireland. J Clin Microbiol 50:4116–4119

Dallman TJ, Byrne L, Ashton PM et al (2015) Whole-genome sequencing for national surveillance of Shiga toxin-producing *Escherichia coli* O157. Clin Infect Dis 61(3):305–312

de Boer RF, Ferdous M, Ott A et al (2015) Assessing the public health risk of Shiga toxin producing *Escherichia coli* by use of a rapid diagnostic screening algorithm. J Clin Microbiol 53:1588–1598

de Souza RL, Carvalhaes JTA, Nishimura LS et al (2011) Hemolytic uremic syndrome in pediatric intensive care units in São Paulo, Brazil. Open Microbiol J 5:76–82

Delannoy S, Mariani-Kurkdjian P, Bonacorsi S et al (2015) Characteristics of emerging human-pathogenic *Escherichia coli* O26:H11 strains isolated in France between 2010 and 2013 and carrying the stx_{2d} gene only. J Clin Microbiol 53:486–492

Duffy G, McCabe E (2014) Veterinary public health approach to managing pathogenic verocytotoxigenic *Escherichia coli* in the agri-food chain. Microbiol Spectr 2(5):EHEC-0023-2013

EFSA Panel on Biological Hazards (BIOHAZ) (2013) Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA J 11(4):3138. http://www.efsa.europa.eu/efsajournal. Accessed 22 Feb 2016

Eppinger M, Mammel MK, Leclerc JE et al (2011) Genomic anatomy of *Escherichia coli* O157:H7 outbreaks. Proc Natl Acad Sci U S A 108(50):20142–20147

European Centre for Disease Prevention and Control (2015) Annual epidemiological report. Food- and waterborne diseases and zoonoses 2014. Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) infection. http://www.ecdc.europa.eu/en/Publications/Annual-Epidemiological-Report-2014.pdf. Accessed 22 Feb 2016

European Centre for Disease Prevention and Control (2015) Expert opinion on the introduction of next-generation typing methods for food- and waterborne diseases in the EU and EEA. http://ecdc.europa.eu/en/publications/Publications/food-and-waterborne-diseases-next-generation-typing-methods.pdf. Accessed 31 March 2016

Feng PCH, Delannoy S, Lacher DW et al (2014) Genetic diversity and virulence potential of Shiga toxin-producing *Escherichia coli* O113:H21 strains isolated from clinical, environmental, and food sources. Appl Environ Microbiol 80(15):4757–4763

Ferdous M, Zhou K, de Boer RF et al (2015) Comprehensive characterization of *Escherichia coli* O104:H4 isolated from patients in the Netherlands. Front Microbiol 6:1348

Ford L, Miller M, Cawthorne A et al (2015) Approaches to the surveillance of foodborne disease: a review of the evidence. Foodborne Pathog Dis 12:927–936

Franz E, van Hoek AHAM, van der Wal FJ et al (2012) Genetic features differentiating bovine, food, and human isolates of Shiga toxin-producing *Escherichia coli* O157 in The Netherlands. J Clin Microbiol 50:772–780

Gadea MP, Deza N, Mota M et al (2012) Two cases of urinary tract infection caused by Shiga toxin-producing *Escherichia coli* O157:H7 strains. Rev Argent Microbiol 44:94–96

Gaulin C, Currie A, Gravel G et al (2014) Summary of 11 years of enteric outbreak investigations and criteria to initiate an investigation, Province of Quebec, 2002 through 2012. J Food Prot 77(9):1563–1570

Gómez SA, Abrey-Recalde MJ, Panek CA et al (2013) The oxidative stress induced in vivo by Shiga toxin-2 contributes to the pathogenicity of haemolytic uraemic syndrome. Clin Exp Immunol 173(3):463–472

Gould LH, Mody RK, Ong KL et al (2013) Increased recognition of non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States during 2000–2010: epidemiologic features and comparison with *E. coli* O157 infections. Foodborne Pathog Dis 10:453–460

Government of Canada (2015) http://healthycanadians.gc.ca/eating-nutrition/risks-recalls-rappels-risques/surveillance/illness-estimates-estimations-maladies/yearly-annuel-eng.php#es. Accessed 1 April 2016

Guth BEC, Picheth CF, Gomes TAT (2010) *Escherichia coli* situation in Brazil. In: Torres AG (ed) Pathogenic *Escherichia coli* in Latin America. Betham Science, Sharjah, pp 162–178

Havelaar AH, Braunig J, Christiansen K et al (2007) Towards an integrated approach in supporting microbiological food safety decisions. Zoonoses Public Health 54(3–4):103–117

Hirai S, Yokoyama E, Etoh Y (2014) Analysis of the population genetics of clades of enterohaemorrhagic *Escherichia coli* O157:H7/H- isolated in three areas in Japan. J Appl Microbiol 117:1191–1197

Ison SA, Delannoy S, Bugarel M et al (2016) Targeted amplicon sequencing for single-nucleotide-polymorphism genotyping of attaching and effacing *Escherichia coli* O26:H11 cattle strains via a high-throughput library preparation technique. Appl Environ Microbiol 82:640–649

Iyoda S, Tamura K, Itoh K et al (2000) Inducible stx_2 phages are lysogenized in the enteroaggregative and other phenotypic *Escherichia coli* O86:HNM isolated from patients. FEMS Microbiol Lett 191:7–10

Jandhyala DM, Thorpe CM, Magun B (2012) Ricin and Shiga toxins: effects on host cell signal transduction. Curr Top Microbiol Immunol 357:41–65

Jenkins C, Dallman TJ, Launders N et al (2015) Public health investigation of two outbreaks of Shiga toxin-producing *Escherichia coli* O157 associated with consumption of watercress. Appl Environ Microbiol 81:3946–3952

Joensen KG, Scheutz F, Lund O et al (2014) Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. J Clin Microbiol 52:1501–1510

Kaper JB, O'Brien AD (2014) Overview and historical perspectives. Microbiol Spectr 2(2):EHEC-0028-2014

Karch H, Tarr PI, Bielaszewska M (2005) Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol 295:405–418

Karmali MA, Mascarenhas M, Shen S et al (2003) Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli s*eropathotypes that are linked to epidemic and/or serious disease. J Clin Microbiol 41:4930–4940

Keen EC (2012) Paradigms of pathogenesis: targeting the mobile genetic elements of disease. Front Cell Infect Microbiol 2:161

Krüger A, Lucchesi PMA (2015) Shiga toxins and *stx* phages: highly diverse entities. Microbiology 161:451–462

Kulasekara BR, Jacobs M, Zhou Y et al (2009) Analysis of the genome of the *Escherichia coli* O157:H7 2006 spinach-associated outbreak isolate indicates candidate genes that may enhance virulence. Infect Immun 77:3713–3721

Lascowski KMS, Guth BEC, Martins FH et al (2013) Shiga toxin-producing *Escherichia coli* in drinking water supplies of north Paraná State, Brazil. J Appl Microbiol 114(4):1230–1239

Launders N, Byrne L, Adams N et al (2013) Outbreak of Shiga toxin-producing *E. coli* O157 associated with consumption of watercress, United Kingdom, August to September 2013. Euro Surveill 18(44). pii: 20624

Lee MS, Kim MH, Tesh VL (2013) Shiga toxins expressed by human pathogenic bacteria induce immune responses in host cells. J Microbiol 51:724–730

Luna-Gierke RE, Griffin PM, Gould LH et al (2014) Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. Epidemiol Infect 142:2270–2280

Manning SD, Motiwala AS, Springman C et al (2008) Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc Natl Acad Sci U S A 105:4868–4873

Matheus-Guimarães C, Gonçalves EM, Guth BEC (2014) Interactions of O157 and non-O157 Shiga toxin–producing *Escherichia coli* (STEC) recovered from bovine hide and carcass with human cells and abiotic surfaces. Foodborne Pathog Dis 11(3):248–255

McGannon CM, Fuller CA, Weiss AA (2010) Different classes of antibiotics differentially influence Shiga toxin production. Antimicrob Agents Chemother 54:3790–3798

McWilliams BD, Torres AG (2014) Enterohemorrhagic *Escherichia coli* adhesins. Microbiol Spectr 2(3):EHEC-0003-2013

Mejias MP, Cabrera G, Jimena Fernández-Brando R et al (2014) Protection of mice against Shiga toxin 2 (Stx2)-associated damage by maternal immunization with a *Brucella* Lumazine Synthase-Stx2 B subunit chimera. Infect Immun 82(4):1491–1499

Mele C, Remuzzi G, Noris M (2014) Hemolytic uremic syndrome. Semin Immunopathol 36:399–420

Melli LJ, Ciocchini AE, Caillava AJ et al (2015) Serogroup-specific bacterial engineered glycoproteins as novel antigenic targets for diagnosis of Shiga toxin-producing-*Escherichia coli*-associated hemolytic-uremic syndrome. J Clin Microbiol 53:528–538

Mellmann A, Harmsen D, Cummings CA et al (2011) Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6(7), e22751

Mellor GE, Sim EM, Barlow RS et al (2012) Phylogenetically related Argentinean and Australian *Escherichia coli* O157 isolates are distinguished by virulence clades and alternative Shiga toxin 1 and 2 prophages. Appl Environ Microbiol 78:4724–4731

Mellor GE, Fegan N, Gobius KS et al (2015) Geographically distinct *Escherichia coli* O157 differ by lineage, Shiga toxin genotype and total Shiga toxin production. J Clin Microbiol 53:579–586

Melton-Celsa AR (2014) Shiga toxin (Stx) classification, structure, and function. Microbiol Spectr 2(3):EHEC-0024-2013

Melton-Celsa AR, O'Brien AD (2014) New therapeutic developments against Shiga toxin-producing *Escherichia coli*. Microbiol Spectr 2(5):EHEC-0013-2013

Miko A, Rivas M, Bentancor A et al (2014) Emerging types of Shiga toxin-producing *E. coli* (STEC) O178 present in cattle, deer and humans from Argentina and Germany. Front Cell Infect Microbiol 4:78

Monecke S, Mariani-Kurkdjian P, Bingen E, Weill FX et al (2011) Presence of enterohemorrhagic *Escherichia coli* ST678/O104:H4 in France prior to 2011. Appl Environ Microbiol 77:8784–8786

Morabito S, Karch H, Mariani-Kurkdjian P et al (1998) Enteroaggregative, Shiga toxin-producing *Escherichia coli* O111:H2 associated with an outbreak of hemolytic-uremic syndrome. J Clin Microbiol 36:840–842

Moxley RA, Acuff GR (2014) Peri- and postharvest factors in the control of Shiga toxin-producing *Escherichia coli* in beef. Microbiol Spectr 2(6):EHEC-0017-2013

Neupane M, Abu-Ali GS, Mitra A et al (2011) Shiga toxin 2 overexpression in *Escherichia coli* O157:H7 strains associated with severe human disease. Microb Pathog 51:466–470

Nyholm O, Halkilahti J, Wiklund G et al (2015) Comparative genomics and characterization of hybrid shigatoxigenic and enterotoxigenic *Escherichia coli* (STEC/ETEC) strains. PLoS One 10(8), e0135936

Paton AW, Paton JC (2010) *Escherichia coli* subtilase cytotoxin. Toxins 2:215–228

Paton AW, Woodrow MC, Doyle R et al (1999) Molecular characterization of a Shiga-toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic-uremic syndrome. J Clin Microbiol 37:3357–3361

Persad AK, LeJeune JT (2014) Animal reservoirs of Shiga toxin-producing *Escherichia coli*. Microbiol Spectr 2(4):EHEC-0027-2014

Pianciola L, Chinen I, Mazzeo M et al (2014) Genotypic characterization of *Escherichia coli* O157:H7 strains that cause diarrhea and hemolytic uremic syndrome in Neuquén, Argentina. Int J Med Microbiol 303:499–504

Pianciola L, D'Astek BA, Mazzeo M et al (2016) Genetic features of human and bovine *Escherichia coli* O157:H7 strains isolated in Argentina. Int J Med Microbiol 306:123–130
Prager R, Lang C, Aurass P et al (2014) Two novel EHEC/EAEC hybrid strains isolated from human infections. PLoS One 9(4), e95379
Riley LW, Remis RS, Helgerson SD et al (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308:681–685
Rivas M, Miliwebsky E, Chinen I et al (2006a) The epidemiology of hemolytic uremic syndrome in Argentina. Diagnosis of the etiologic agent, reservoirs and routes of transmission. Medicina (Buenos Aires) 66:27–32
Rivas M, Miliwebsky E, Chinen I et al (2006b) Characterization and epidemiologic subtyping of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina. Foodborne Pathog Dis 3:88–96
Rivas M, Chinen I, Miliwebsky E et al (2014) Risk factors for Shiga toxin-producing *Escherichia coli*-associated human diseases. Microbiol Spectr (5):EHEC-0002-2013
Sandvig K, Bergan J, Kavaliauskiene S et al (2014) Lipid requirements for entry of protein toxins into cells. Prog Lipid Res 54:1–13
Scheutz F (2014) Taxonomy meets public health: the case of Shiga toxin-producing *Escherichia coli*. Microbiol Spectr 2(4):EHEC-0019-2013
Scheutz F, Teel LD, Beutin L et al (2012) Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J Clin Microbiol 50:2951–2963
Schüller S (2011) Shiga toxin interaction with human intestinal epithelium. Toxins 3(6):626–639
Siegler RL, Obrig TG, Pysher TJ et al (2003) Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. Pediatr Nephrol 18:92–96
Smith DR (2014) Vaccination of cattle against *Escherichia coli* O157:H7. Microbiol Spectr 2(6):EHEC-0006-2013. doi:10.1128/microbiolspec.EHEC-0006-2013
Spinale JM, Ruebner RL, Copelovitch L et al (2013) Long-term outcomes of Shiga toxin hemolytic uremic syndrome. Pediatr Nephrol 28:2097–2105
Stearns-Kurosawa DJ, Collins V, Freeman S et al (2010) Distinct physiologic and inflammatory responses elicited in baboons after challenge with Shiga toxin type 1 or 2 from enterohemorrhagic *Escherichia coli*. Infect Immun 78(6):2497–2504
Stearns-Kurosawa DJ, Oh SY, Cherla RP et al (2013) Distinct renal pathology and a chemotactic phenotype after enterohemorrhagic *Escherichia coli* Shiga toxins in non-human primate models of hemolytic uremic syndrome. Am J Pathol 182(4):1227–1238
Szu SC, Ahmed A (2014) Clinical studies of *Escherichia coli* O157:H7 conjugate vaccines in adults and young children. Microbiol Spectr 2(6):EHEC-0016-2013
Tanaro JD, Piaggio MC, Galli L et al (2014) Prevalence of *Escherichia coli* O157:H7 in surface water near cattle feedlots. Foodborne Pathog Dis 9(11):960–965
Tarr PI, Gordon CA, Chandler WL (2005) Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 365:1073–1086
Terajima J, Iyoda S, Ohnishi M et al (2014) Shiga toxin (verotoxin)-producing *Escherichia coli* in Japan. Microbiol Spectr 2(5):EHEC-0011-2013
Tozzoli R, Ardissino G, Torresani E et al (2015) Characterization of an enteroaggregative-Shiga toxin producing *Escherichia coli* O127:H4 strain from an outbreak in a primary school in Northern Italy. In: Abstracts of the ninth triennial international symposium on Shiga toxin-producing *Escherichia coli* infections, Boston, 13–16 Sept 2015
Vally H, Hall G, Dyda A et al (2012) Epidemiology of Shiga toxin producing *Escherichia coli* in Australia, 2000–2010. BMC Public Health 12:63
Varela G, Schelotto F (2015) Síndrome urémico hemolítico en Uruguay. Aspectos microbiológicos y clínicos, aportes para su conocimiento regional. Rev Fac Cienc Salud UDES 2:25–30. doi: 10.20320/rfcsudes-201521-416
Whitney BM, Mainero C, Humes E et al (2015) Socioeconomic status and foodborne pathogens in Connecticut, USA, 2000-2011. Emerg Infect Dis 21:1617–1624

Whitworth J, Zhang Y, Bono J et al (2010) Diverse genetic markers concordantly identified bovine origin *Escherichia coli* O157 genotypes underrepresented in human disease. Appl Environ Microbiol 76:361–365

Wick LM, Qi W, Lacher DW et al (2005) Evolution of genomic content in the stepwise emergence of *Escherichia coli* O157:H7. J Bacteriol 187:1783–1791

Wickham ME, Lupp C, Mascarenhas M et al (2006) Bacterial genetic determinants of non-O157 STEC outbreaks and hemolytic-uremic syndrome after infection. J Infect Dis 194:819–827

Yang Z, Kovar J, Kim Jet AL (2004) Identification of common subpopulations of non-sorbitol-fermenting, β-glucuronidase-negative *Escherichia coli* O157:H7 from bovine production environments and human clinical samples. Appl Environ Microbiol 70:6846–6854

Zoja C, Buelli S, Morigi M (2010) Shiga toxin-associated hemolytic uremic syndrome: pathophysiology of endothelial dysfunction. Pediatr Nephrol 25(11):2231–2240

Chapter 6
Diffusely Adherent *Escherichia coli*

Mario Meza-Segura and Teresa Estrada-Garcia

Summary Diffusely adherent *Escherichia coli* (DAEC) were the last diarrheagenic *E. coli* pathogroup (DEC) to be recognized. DAEC pathogroup encompasses a heterogeneous group of *E. coli* strains, harboring genes encoding for Afa/Dr adhesins that are capable of causing diarrhea illness in otherwise healthy individuals. Afa/Dr family includes Afa, Dr, and F1845 adhesins that are encoded by the *afa*/*dra*/*daa* operons, respectively. Afa/Dr adhesins bind to cell receptors human decay-accelerating factor (hDAF) and carcinoembryonic antigen cell adhesion molecules (hCEACAMs), inducing receptor clustering and finger-like cell projections, resulting in bacteria embedding without complete internalization. DAEC infection also causes damages on epithelial cells, including loss of microvilli structure, impaired enzyme activities of functional brush border-associated proteins, and loss of adherens junctions, in an Afa/Dr-dependent fashion. Diarrhea prevalence studies among Latin American children have confirmed that DAEC diarrhea illness susceptibility is age-related and DAEC-diarrhea risk increases with children's age. DAEC was the most prevalent DEC identified from children with acute diarrhea attending a hospital rehydration unit and an Emergency Room service in Mexico and US, respectively. In adults, DAEC was identified in travelers' diarrhea patients who visited Latin America and from HIV-positive patients with diarrhea from Peru. Recently, DAEC strains carrying virulence genes associated with pathogenesis, M cell translocation, angiogenesis, and genotoxicity were isolated from patients with inflammatory bowel disease and colorectal cancer. DAEC is a bacterial pathogen that induces unique alterations on epithelial cells, resulting in diarrhea illness and epithelia damage that may also contribute to the development of other intestinal diseases.

M. Meza-Segura • T. Estrada-Garcia (✉)
Department of Molecular Microbiology, CINVESTAV-IPN,
AV. IPN No.2508, Col. Zacatenco, Mexico City 07360, Mexico
e-mail: mmeza@cinvestav.mx; testrada@cinvestav.mx

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_6

1 General Concepts

Diffusely adherent *Escherichia coli* (DAEC) strains are defined by the presence of a characteristic diffuse adherence (DA) pattern on HeLa and HEp-2 epithelial cells (Scaletsky et al. 1984; Nataro et al. 1985). DA pattern consists of bacteria being uniformly adhered all over the entire surface of epithelial cells (Fig. 6.1a). Based on the expression of adhesins, two groups of DAEC strains were identified, Afa/Dr DAEC and AIDA-I DAEC; moreover, these adhesins are responsible for the DA phenotype. Nonetheless, not all *E. coli* strains exhibiting the DA pattern carry Afa/Dr or AIDA-I adhesins (Scaletsky et al. 2002c). Further characterization of DAEC strains expressing AIDA-I adhesins revealed that these isolates also harbor the virulence gene encoding for intimin (*eae*) that defines strains belonging to the atypical enteropathogenic *E. coli* (EPEC) pathogroup (Beinke et al. 1998; Servin 2014). Although *afa*/*dra*-positive uropathogenic *E. coli* (UPEC) strains have been identified, these adhesins are not among the main virulence factors involved in UPEC pathogenesis (Flores-Mireles et al. 2015). In contrast, Afa/Dr DAEC strains have been associated with acute diarrhea in children, particularly in children ≥6 months of age and with persistent diarrhea (Baqui et al. 1992; Levine et al. 1993; Germani et al. 1996; Spano et al. 2008; Ochoa et al. 2009a; Lozer et al. 2013; Mansan-Almeida et al. 2013; Patzi-Vargas et al. 2015). Consequently in 1998, DAEC was recognized as the sixth class of diarrheagenic *E. coli* (Nataro and Kaper 1998). Therefore, DAEC pathogroup encompasses a heterogeneous group of *E. coli* strains, harboring genes encoding for Afa/Dr adhesins that are capable of causing diarrheal illness in otherwise healthy individuals.

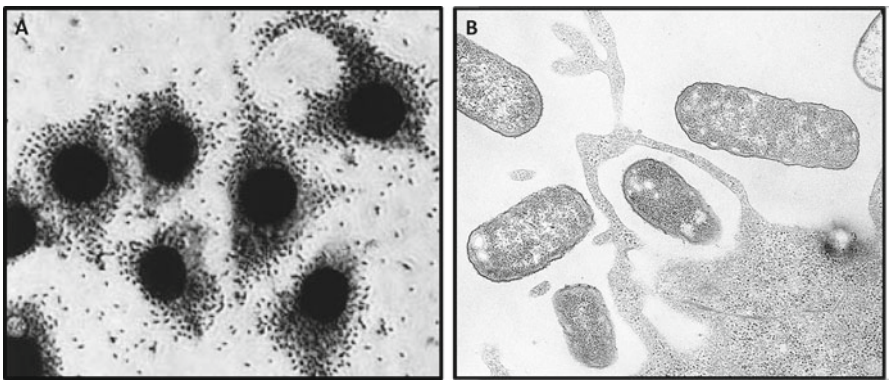

Fig. 6.1 DAEC adhesion to epithelial cells: (**a**) Diffusely Adherent pattern on HeLa cells. (**b**) Transmission electron microscopy showing finger-like projections extending from the surface of HEp-2 cells induced by C1845 infection. Taken from Arikawa et al. (2005) and Cookson and Nataro (1996)

The Afa/Dr family of adhesins includes fimbrial (Dr, F1845) and afimbrial adhesins (Afa) that attach to the Dr blood group antigen, a component of the Cromer-related blood complex, inducing hemagglutination (Nowicki et al. 2001). After Afa/Dr adhesins bind to their cell membrane receptors, the human decay-accelerating factor (hDAF) and the human carcinoembryonic antigen cell adhesions molecules (hCEACAMs), they induce receptor clustering and activation of signaling pathways, promoting structural modifications on the intestinal barrier and inducing pro-inflammatory responses (Servin 2014).

2 DAEC Virulence Factors and Pathogenesis

DAEC pathogenesis initiates as most pathogens with the adhesion to intestinal epithelial cells. *Ex vivo* experiments have shown that DAEC strains adhere to human small intestine, exhibiting a better adherence to M cells micro-folds than to microvilli of Peyer's patch-associated epithelium (Yamamoto et al. 1992). Moreover, DAEC has also the ability to colonize human undifferentiated crypt cells and differentiated enterocytes (Kerneis et al. 1991). DAEC attachment onto the target host cells induces finger-like projections extending from the surface of infected epithelial cells (Caco-2 or HEp-2), thus embedding the bacteria without complete internalization in the cell (Fig. 6.1b). DAEC embedding occurrence may provide protection against host-mediated defense mechanisms and antibiotics, as well as resistance to intestinal clearance by peristalsis (Yamamoto et al. 1994; Cookson and Nataro 1996).

Afa/Dr adhesins expressed on DAEC outer membrane are responsible for both, DA pattern on epithelial cells and adherence to the intestinal epithelium. This family encompasses fimbrial and afimbrial adhesins. The former are filamentous appendages, while the latter are composed of non-covalently bound subunits exhibiting a capsule-like appearance (Duguid et al. 1955; Goldhar 1996). Afa/Dr adhesins are exported across the cytoplasmic membrane via a general secretory pathway (GSP) and then escorted to the surface via a periplasmic chaperone/usher machinery. The periplasmic chaperone facilitates subunits folding and their delivery to the usher (an outer-membrane pore-forming protein), which acts as an assembly platform for subunits polymerization (Fig. 6.2) (Zav'yalov et al. 2010).

Afa-I, Afa-II, Afa-III, Afa-V, Afa-VII, Afa-VIII, and Dr-2 afimbrial adhesins, as well as Dr and F1845 fimbrial adhesins, constitute the Afa/Dr family (Table 6.1). Most of these adhesins have been identified in *E. coli* strains isolated from human urinary tract infections or diarrhea cases, with the exception of Afa-VII, which has only been found in *E. coli* isolates from bovine feces (Lalioui et al. 1999). F1845 adhesin was first identified in an *E. coli* strain (C1845) isolated from a child with persistent diarrhea (Bilge et al. 1989). To date, only the genes encoding for Afa-I, Afa-II, Afa-III, and Afa-V have been identified in *E. coli* strains isolated from diarrheal cases (Table 6.1) (Servin 2014).

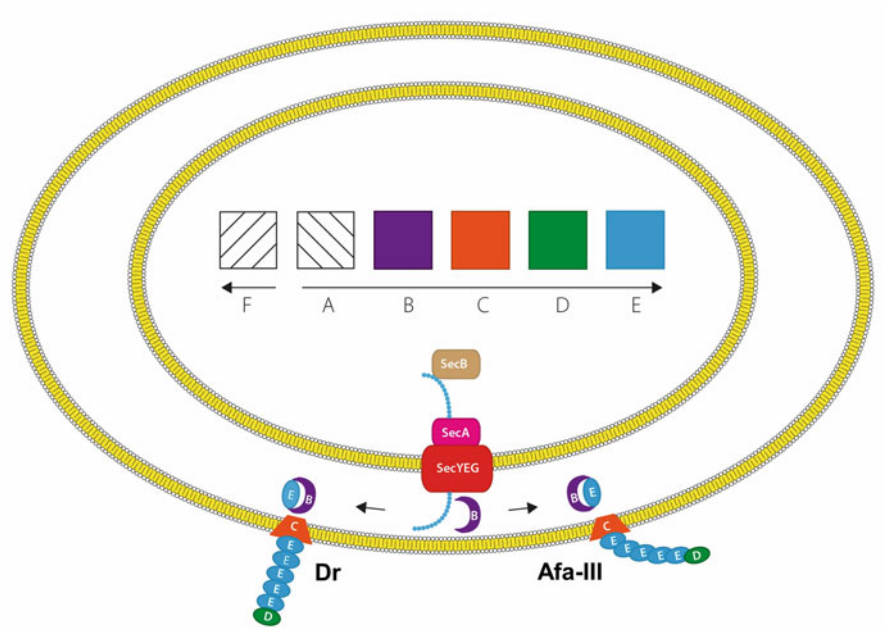

Fig. 6.2 General genetic organization of *afa*/*dra*/*daa* operons and assembly of Dr and Afa-III adhesins: *afa*/*dra*/*daa* operons include five genes: A, B, C, D, E, encoding for a transcriptional regulator, a chaperone, an usher, an invasion, and an adhesin structural subunit, respectively. *afa-3*/*daa* operons are also regulated by F subunits encoded on divergent transcriptional units. Once D or E subunits are produced, they are transported to the periplasm via the general secretory pathway. The periplasmic chaperon facilitates subunits folding and their delivery to the usher, which transports subunits through the outer membrane and acts as an assembly platform for their polymerization. D subunit is localized at the tip of the E subunits structure. Dr fimbriae and Afa-III adhesin suggested structures are illustrated. Dr fimbriae is a rigid structure, while Afa-III seems to be flexible due to its fine filaments

Afa, Dr, and F1845 adhesins are encoded by the *afa*/*dra*/*daa* operons, respectively. All these operons contain at least five different genes with a conserved distribution and function, A, B, C, D, and E, encoding for a transcriptional regulator, a chaperone, an usher, an invasion, and an adhesin structural subunit, respectively (Fig. 6.2) (Servin 2014). *afa*/*dra*/*daa* A, B, C, and D genes are highly conserved, while among most members of this family the E gene is less conserved; except for *afaE-3* and *draE*, which have a 99 % homology and a 98 % identity (Labigne-Roussel and Falkow 1988; Le Bouguenec et al. 1993; Garcia et al. 2000). Therefore, most AfaE/DraE/DaaE subunits are antigenically diverse (Labigne-Roussel and Falkow 1988). It has been shown that *afa-3* and *daa*, two of the best characterized Afa/Dr adhesin operons, are regulated by *afaF*/*daaF* gene products, harbored in divergent transcriptional units (Bilge et al. 1993; Garcia et al. 1994). Although Afa adhesins were initially described as afimbrial proteins, nuclear magnetic resolution studies revealed that Afa-III has a fimbrial structure made of several AfaE subunits and

Table 6.1 General characteristics of Afa/Dr adhesins

Adhesin	Operon	Type	Identified cellular receptor(s)	Isolated from	Reference strain
AfaE-I	*afa-1*	A/F[a]	DAF, CEA	D, UTI	KS52
AfaE-II	*afa-2*	A	DAF	D, UTI	A22
AfaE-III	*afa-3*	A/F	DAF, CEACAM1, CEA, CEACAM6	D, UTI	A30
AfaE-V	*afa-5*	A	DAF, CEA	D, UTI	AL 851
AfaE-VII	*afa-7*	A	Unknown	BO	262 KH 89
AfaE-VIII	*afa-8*	A	Unknown	B, BO, PO, UTI	239 KH 89, Al 862
Dr	*dra*	F	DAF, CEACAM1, CEA, CEACAM6, collagen type-4[b]	UTI	IH11128
Dr-II	*dra-2*	A	DAF	UTI	EC7372
F1845	*daa*	F	DAF, CEACAM1, CEA, CEACAM6	D, UTI	C1845

DAEC diffusely adherent *E. coli*, *B* blood, *BO* bovine, *D* diarrhea, *PO* porcine, *UTI* urinary tract infections, *A* afimbrial, *F* fimbrial, *DAF* decay-accelerating factor, *CEA* carcinoembryonic antigen, *CEACAM* carcinoembryonic antigen-related cellular adhesion molecule

[a]AfaE-I has been reported as both, an afimbrial (Labinge-Roussel et al. 1984) and a fimbrial adhesin (Keller et al. 2002)

[b]Only Dr recognized collagen type-4 (Carnoy and Moseley 1997)

capped by one AfaD subunit (Anderson et al. 2004). Afa adhesin fine fibrillar structures may be collapsed onto the bacterial surface explaining why they were initially described as afimbrial adhesins (Fig. 6.2).

3 Host Cell Receptors for Afa/Dr Adhesins

3.1 Human Decay-Accelerating Factor

The human cell surface decay-accelerating factor (hDAF, CD55) is recognized by most members of the Afa/Dr family, with the exception of Afa-VII and Afa-VIII, receptors which have not yet been characterized (Nowicki et al. 1993, 2001; Lalioui et al. 1999). Afa/Dr-hDAF recognition seems to be species-specific, since these adhesins do not recognize guinea pig, rat, mice, or pig DAF receptors (Hudault et al. 2004). The hDAF is a 70 kDa glycoprotein; the recognition region contains four short complement control protein repeat (CCP) domains (of ~60 amino acids each), followed by a 67-amino acid middle region, rich in serine/threonine/proline (STP), amino acids heavily O-glycosylated, and the cell membrane bound region formed by a carboxyl-terminal glycosylphosphatidylinositol (GPI) anchor (Lublin 2005). The physiological function of hDAF is to control the complement-cascade amplification through a direct interaction with membrane-bound C3b or C4b molecules, resulting

in the inhibition of the downstream complement cascade, known as the decay-accelerating activity. hDAF is expressed on the surface of peripheral blood cells, endothelial cells, and epithelial cells (Lublin 2005). Afa/Dr adhesins bind to CCP-2 and CCP-3 epitopes dissimilar to those recognized by complement molecules (Nowicki et al. 1993; Guignot et al. 2000; Selvarangan et al. 2000; Hasan et al. 2002). Binding of Afa/Dr adhesins to hDAF receptor induces hDAF clustering around adhering bacteria, a process requiring c-Src kinase activation and the presence of hDAF CCP1 domain (Guignot et al. 2000; Bétis et al. 2003a, b; Queval et al. 2011) (Fig. 6.3).

Infection of human epithelial T84 cell monolayers with DAEC C1845 or UPEC IH11128 (Dr positive strain) induces interleukin (IL)-8 secretion through hDAF-dependent activation of ERK1/2, P38, and JNK; members of the mitogen-activated protein kinases (MAP kinases) (Bétis et al. 2003a). IL-8 secretion results in polymorphonuclear leucocyte (PMNL) transepithelial migration, which induces tumor necrosis factor (TNF)-α and IL-1β production, cytokines that upregulate hDAF expression and clustering around adhering bacteria (Bétis et al. 2003a, b). Moreover, DAEC strains isolated from patients increase the production of IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF in CaCo-2 cells when compared with a commensal *E. coli* isolate (Patzi-Vargas et al. 2013). It has been shown that most of these cytokines upregulated hDAF expression on epithelial cells (Andoh et al. 1996, 1997).

3.2 Human Carcinoembryonic Antigen-Related Cell Adhesion Molecules

Some Afa/Dr family members bind to human carcinoembryonic antigen-related cell adhesion molecules (hCEACAM), a group of mammalian immunoglobulin-related glycoproteins, involved in intercellular adhesion and regulation of cell signaling activities (Table 6.1) (Berger et al. 2004; Kuespert et al. 2006). The hCEACAMs are expressed on epithelial, endothelial, and hematopoietic cells and have been implicated in inflammation, immune responses, angiogenesis, apoptosis, cancer, and in cell recognition by virus and bacterial pathogens (Kuespert et al. 2006; Beauchemin and Arabzadeh 2013). Afa-I and Afa-V adhesins only bind to hCEA, while AfaE-III, Dr, and F1845 each can bind to hCEACAM1, hCEA and hCEACAM6 (Berger et al. 2004; Korotkova et al. 2006). The hCEACAM1 and hCEACAM3 are anchored via transmembrane domains, whereas hCEA and hCEACAM6 through a GPI linkage (Beauchemin and Arabzadeh 2013). F1845, AfaE-III, and Dr adhesins also induce recruitment of hCEACAM1, hCEACAM3, hCEA, and hCEACAM6 receptors (Guignot et al. 2000; Berger et al. 2004). Furthermore, bacteria binding to hCEA and hCEACAM6, but not hCEACAM1, promotes cell membrane finger-like extensions around the attached bacterium (Fig. 6.3). Finger-like extensions are mediated by actin-binding proteins ezrin/radixin/moesin (ERM) (Berger et al. 2004). It has been reported that at least AfaE-I, AfaE-V, DraE, and DaaE adhesins can bind simultaneously to both hDAF and hCEA receptors (Korotkova et al. 2006).

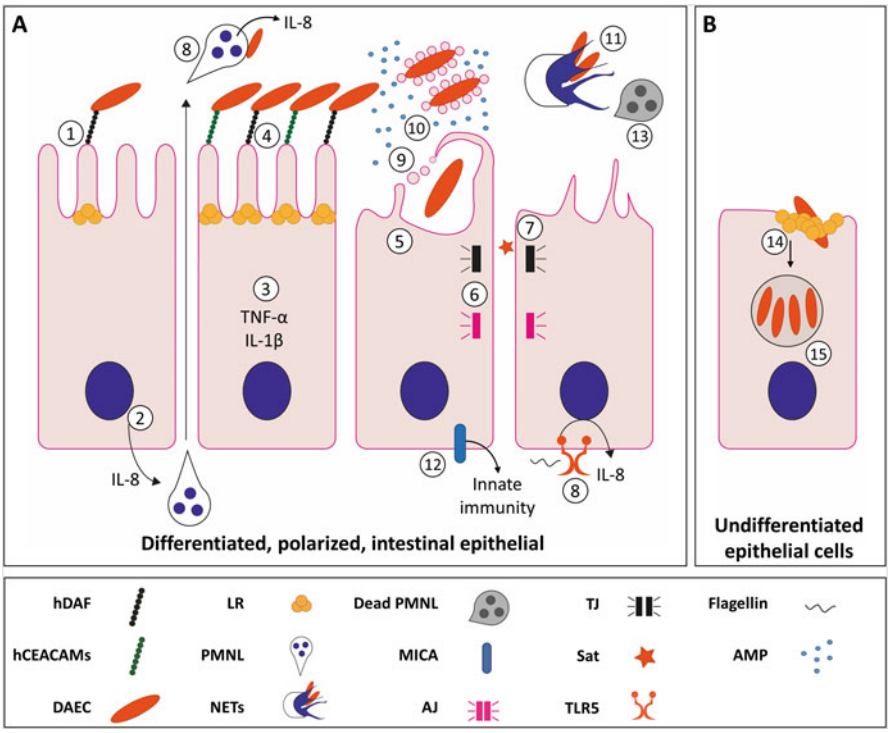

Fig. 6.3 DAEC infection. (**a**) hDAF and hCEACAMs receptors on the surface of intestinal epithelial cells are recognized by Afa/Dr adhesins (1). DAEC infection elicits IL-8 secretion resulting in migration of polymorphonuclear leucocytes (PMNL) (2). PMNL migration induces TNF-α and IL-1β cytokine production (3), that upregulate hDAF expression. hDAF and hCEACAMs clustering is induced accompanied by lipid rafts (LR) mobilization (4). DAEC infection causes damages on epithelial cells, including loss of microvilli structure (5), impaired enzyme activities of functional brush border-associated proteins, and loss of adherens junctions (AJ) (6). In addition, the secreted autotransporter toxin (Sat) induces damages on epithelial cells and alters tight junction (TJ) integrity (7). Bacterial type 1 pili and flagellin trigger a secondary IL-8 production after binding to PMNLs and TLR5, respectively (8). On the other hand, enterocyte cells elicit protective responses against DAEC infection, including the release of microvilli tips underneath attached bacteria (9), secretion of antimicrobial peptides (AMP) (9), release of neutrophil extracellular traps (NETs) (11), and expression of MICA molecules that may activate innate immune responses against infection (12). However, infection of PMNL with DAEC reduces their phagocytic capacity and induces apoptosis (13). (**b**) Undifferentiated epithelial cells are invaded by DAEC. The process of internalization requires lipid rafts integrity and dynamic unstable microtubules (14). Two hypotheses have been proposed. The first one suggests that AfaD/DraD invasin subunits recognize the membrane-bound α5β1 integrin and trigger bacteria entry. The second hypothesis proposes an AfaE/DraE-mediated internalization through hDAF or hCEACAM interaction. Once inside, DAEC forms inclusions in which they are able to survive for at least 72 h (15)

4 Afa/Dr DAEC Invasion Capacity

DAEC strains have a low level invasive capacity of human non-polarized epithelial cells expressing Afa/Dr adhesin receptors (Goluszko et al. 1997; Jouve et al. 1997; Guignot et al. 2001), whereas they are not able to invade intact human-polarized intestinal cell monolayers, except when intercellular junctions are disrupted (Guignot et al. 2001; Plançon et al. 2003; Kansau et al. 2004). The process of internalization into non-polarized epithelial cells requires lipid rafts integrity (Selvarangan et al. 2000; Guignot et al. 2001, 2009; Kansau et al. 2004; Korotkova et al. 2008) and dynamic unstable microtubules (Goluszko et al. 1997; Guignot et al. 2001, 2009; Korotkova et al. 2008). Once inside, DAEC forms inclusions in which they are able to survive for at least 72 h (Plançon et al. 2003).

Different authors have attempted to elucidate the processes by which DAEC strains invade epithelial cells. However, this process remains controversial and at least two hypotheses have been proposed. The first one implies that AfaD/DraD invasin subunits recognize the membrane-bound α5β1 integrin and that this interaction is sufficient to trigger bacterial entry via a zipper-like mechanism, which is independent of Afa/Dr-induced F-actin mobilization, but dependent of cholesterol (Jouve et al. 1997; Guignot et al. 2001; Plançon et al. 2003; Kansau et al. 2004). In accordance, recruitment of β1 integrin, lipid rafts, and caveolin has been observed during hDAF-Afa/Dr interaction (Kansau et al. 2004). The second hypothesis proposes that AfaD/DraD invasin subunits are not required for bacterial adhesion or internalization. Instead, hDAF or hCEACAM receptors independently promote bacteria DraE-mediated internalization, dependent of lipid rafts integrity, microtubules, and phosphatidylinositol 3-kinase (PI3K) activity, but once more independent of F-actin (Goluszko et al. 1997; Selvarangan et al. 2000; Das et al. 2005; Korotkova et al. 2008; Guignot et al. 2009).

On the other hand, it seems that the presence of both AfaD and AfaE subunits is necessary for the invasion process (Fig. 6.3). In as much as only when AfaD and AfaE were coupled (AfaDE) and attached to glass beats, it was possible to reproduce all the previously described events: attachment, β1 integrin clustering beneath cell-associated beads, and invasion (Cota et al. 2006). Furthermore, these authors reported a low affinity of AfaDE for α5β1 integrin when compared with surface proteins of truly invasive bacteria; explaining the low level invasive capacity of Afa/Dr bacteria (Cota et al. 2006).

5 Virulence Factors

5.1 Flagella

DAEC infection induces high levels of IL-8 secretion in epithelial cells (Arikawa et al. 2005; Bétis et al. 2003a, b; Patzi-Vargas et al. 2013). However, diffusive adhesiveness itself is unlikely to be sufficient to promote this effect (Arikawa and

Nishikawa 2010). Several studies documented that DAEC mobile strains induce high levels of IL-8 secretion (Arikawa and Nishikawa 2010; Tanimoto et al. 2013). In line, purified flagella from Afa/Dr DAEC was able to induce IL-8 production in a TLR5 fashion. Because TLR5 is exclusively found on the basolateral membrane of polarized epithelial cells, motile DAEC isolates must loosen tight junctions (TJ) for flagella to reach TLR5 and trigger signaling that results in the induction of IL-8 (Arikawa and Nishikawa 2010; Tanimoto et al. 2013). Furthermore, DAEC strains that increased FITC-dextran paracellular passage and altered transepithelial resistance (TER) of infected epithelial cells also induced high levels of IL-8 secretion (Tanimoto et al. 2013). This suggests that DAEC possess supplementary factors, in addition to Afa/Dr adhesin and flagellin, which can induce high levels of IL-8 secretion.

5.2 Sat

F1845-independent alterations of TJ proteins, occludin and ZO-1, were observed in Caco-2/TC7 cell monolayers infected with C1845 strain, in conjunction with an increase in paracellular permeability without a decrease of TER (Peiffer et al. 2000a). Similar results were observed with polarized epithelial Caco-2/TC7 cells infected with Afa/Dr UPEC IH11128 (Guignot et al. 2007). Overall, these experiments suggested that Afa/Dr-hDAF interaction was not sufficient for inducing structural and functional TJ lesions. Caco-2/TC7 cells infected with bacteria transfected with a putative virulence factor identified in IH11128 strain exhibited similar alterations than when infected with IH11128 strain (Guignot et al. 2007). Sequence homology analysis revealed that the putative virulence factor is the secreted autotransporter toxin (Sat). In addition to Sat effects on TJ integrity, other effects as vacuolating cytotoxin activity, and impairment of brush border-associated sucrase-isomaltase (SI) and dipeptidyl peptidase IV (DPP IV) enzyme activity on epithelial cells, have been described (Peiffer et al. 2000b, 2001; Dautin 2010).

Sat belongs to the family of serine protease autotransporters of *Enterobacteriaceae* (SPATE), which includes a variety of virulence toxins such as Pet, Pic, EspC, SigA, SepA, Tsh, and EspP (Dautin 2010). Sat-induced disassembly of TJs-associated proteins is dependent on its serine protease motif (GDSDSG), as for Pet and EspC (Dautin 2010). Alterations of TJs-associated proteins are more pronounced when cells are infected with *E. coli* strains expressing both Sat and the Dr adhesin, suggesting that the delivery of Sat at the vicinity of the cell membrane could allow more efficient binding of Sat (Guignot et al. 2007). Sat has also enterotoxic activity in rabbit ileal loops, hence induced a pronounced fluid accumulation, villous necrosis, submucosal edema and polymorphonuclear lymphocytes (PMNL) infiltration, to a similar extent than those effects produced by heat-labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) (Taddei et al. 2005). Prevalence of the *sat* gene among DAEC strains was significantly higher than in other pathogroups (Taddei et al. 2003). In two independent studies, a 46 % *sat* prevalence was observed among DAEC strains collected from children with diarrhea compared with a 16 % and 18.9 %, respectively, in DAEC strains from asymptomatic children (Guignot et al. 2007; Mansan-Almeida

et al. 2013). Moreover, *sat* has also been identified in C1845 (F1845) and AL 851 (AfaE-V) reference strains; both strains were isolated from stools of children with diarrhea (Guignot et al. 2007). Therefore, it seems that *sat* is more prevalent among pathogenic DAEC strains.

5.3 Other Virulence Factors

In addition, it has been reported that some DAEC strains collected from children with diarrhea carry genes homologous to those encoding for certain molecules of EPEC and enterohemorrhagic *E. coli* (EHEC) type three-secretion system (T3SS), and moreover, some of these DAEC strains actually can produce EspA, EspB, and EspD homolog proteins (Beinke et al. 1998; Kyaw et al. 2003; Mansan-Almeida et al. 2013). In line, some DAEC strain also induced the formation of pedestals and extended surface structures, accompanied by accumulation of actin and tyrosine-phosphorylation of proteins underneath the attached bacteria (Beinke et al. 1998). DAEC strains harboring genes encoding for type 1 pili have been described (Lopes et al. 2005; Prorok-Hamon et al. 2014). It has been demonstrated that some DAEC strains elicit a late IL-8 production by PMNL (through activation of Src and the MAPK), in a type-1 pili-dependent manner (Sémiramoth et al. 2009). Curli fimbriae as well is present in some DAEC strains. Curli has been associated with higher rates of *E. coli* invasion to epithelial cells, increased *E. coli* virulence in mice models, and induction of inflammatory responses mediated by TLR1/TLR2 (Barnhart and Chapman 2006; Tükel et al. 2009; Mansan-Almeida et al. 2013). Furthermore, curli was more frequently expressed in DAEC strains isolated from adults with diarrhea (59.2 %) than from asymptomatic carriers (6.7 %) (Mansan-Almeida et al. 2013). Finally, the presence of other virulence factors genes in DAEC strains has been documented: including toxins (*astA*), siderophores (*fyuA*, *irp2*, *iuc, and iroN*), transport systems (*shu* and *modD*), and intra-macrophage survival factors (*htrA* and *dsbA*) (Blanc-Potard et al. 2002; Kyaw et al. 2003; Lopes et al. 2005; Mansan-Almeida et al. 2013; Prorok-Hamon et al. 2014; Patzi-Vargas et al. 2015).

6 Host Cell Responses Against DAEC Infection

Enterocyte cells elicit protective responses against pathogens to prevent intestinal infections. The most immediate responses include the secretion of antimicrobial peptides and inflammatory mediators. Intestinal epithelial cells express gut antimicrobial components in a cell differentiation-dependent manner, as lysozyme, α-antitrypsin, PR-39, and cecropin P1 (Bernet-Camard et al. 1996a). Production of antimicrobial components by host cells correlates with C1845 bacteria damages, characterized by bacteria flattening, multiple surface bundle, and a significant decrease of viable bacteria after 3 h of incubation (Bernet-Camard et al. 1996a).

Furthermore, it has been reported that after infection with DAEC strains, the actin network of intestinal cells is disassembled, releasing microvilli tips underneath attached bacteria (Bernet-Camard et al. 1996b). Releasing of microvilli tips correlates with cell differentiation status, since it is not observed when bacteria are incubated with undifferentiated cells. Detached vesicles made of microvillus membranes allow the extrusion of brush border-colonizing bacteria and probably inhibit the adhesion of luminal bacteria to epithelial cells (Shifrin et al. 2013).

DAEC infection of epithelial cells induces IL-8 secretion, promoting neutrophils chemotaxis to the infection site (Bétis et al. 2003b; Arikawa and Nishikawa 2010; Tanimoto et al. 2013). In turn, neutrophils play an important role in bacterial elimination, as it has been described for the release of neutrophil extracellular traps (NETs), composed of a nuclear DNA backbone associated with antimicrobial peptides, histones, and proteases, which entrap and kill pathogens as for C1845 DAEC strain (Brinkmann and Zychlinsky 2012; Marin-Esteban et al. 2012). On the other hand, DAEC strains diminish PMNL phagocytic capacity and induce their apoptosis (Brest et al. 2004). C1845-infected PMNL, even in the absence of apoptotic markers, are removed by macrophages, a process that may be involved in the resolution of DAEC infection and reduction of inflammation responses (Sémiramoth et al. 2010). AfaE-I and AfaE-III adhesion to hDAF induces the expression of the major histocompatibility complex (MHC) class I-related molecules A (MICA) by intestinal epithelial cells (Tieng et al. 2002). MICA are rapidly recognized by the NKG2D receptor, which is expressed on the surface of NK cells, γδ T cells, and CD8+ αβ T cells (Bauer et al. 1999). Thus, it is possible that MICA expression induced by DAEC infection may act as a danger signal enhancing innate immune responses against pathogens.

7 DAEC Detection

Because EPEC and Afa/Dr negative *E. coli* can as well produce a DA pattern on HEp-2 and HeLa cells, consequently cell adhesion assays are unsuitable for identification of DAEC strains harboring Afa/Dr adhesins (Hernandes et al. 2009; Scaletsky et al. 2002c).

Therefore, we will only discuss molecular methods targeting genes or operon regions of the *afa* and *daa* operons. As for most molecular DEC characterization studies, DAEC identification was initiated by the use of DNA probes (Bilge et al. 1989). During the years, several probes were developed, *drb* a 260-bp fragment coding for AfaE-I adhesin of UPEC KS52 as well as two *daaC* probes: a 300-bpI *Pst*I fragment from *daa* operon, cloned into plasmid pSS1, and ~390–370-bp *Pst*I fragment, cloned into plasmid pSLM852 (Bilge et al. 1989; Smith et al. 1994; Scaletsky et al. 2002a). The specificity of the latter *daaC* probe was questioned very soon, since it was reported that 93 % of 86 molecularly characterized enteroaggregative *E. coli* (EAEC) strains also hybridized with this probe (Smith et al. 1994). Snelling and colleagues reported as well that *daaC* probe (pSLM852) cross-hybridizes with a sub-set of EAEC strains, including some test and reference strains, including 042 the

prototypical EAEC strain (Snelling et al. 2009). The authors revealed that the cross-hybridization is due to 84 % identity at the nucleotide level, between the *daaC* locus and the fimbriae II cluster gene, *aafC*, present in some EAEC strains. Alongside, the 419 bp DNA fragment homologous to *daaE* probes that identifies F1845 fimbria is not as well suitable for Afa/Dr adhesin identification since the prevalence of F1845 fimbria among *E. coli* isolates from stool is rare (Campos et al. 1999).

On the other hand, specific sequence probes MO30, S109, and S111 for C1845 strain were developed by DNA subtraction analysis between C1845 and K12 *E. coli* strains. It has been shown that the MO30 probe is highly prevalent among Afa/Dr strains including UPEC and DAEC, but did not hybridized with any ETEC, EAEC, or EPEC isolates (Blanc-Potard et al. 2002; Escobar-Páramo et al. 2004).

PCR methods are more specific than DNA probes for DEC identification (Scaletsky et al. 2002a; Meraz et al. 2008). In recent years, single and multiplex PCR methods have been developed for DEC identification, including DAEC (López-Saucedo et al. 2003; Meraz et al. 2008; Patzi-Vargas et al. 2013).

8 Recent Advances in DAEC Research

Recently, it was reported that DAEC strains carrying the long polar fimbriae (*lpf*) gene and the polyketide synthase gene complex (*pks*) are common in inflammatory bowel disease (IBD) and in colorectal cancer (CRC) (Prorok-Hamon et al. 2014). Lpf was first described in EHEC as a protein involved in translocation across M cells of the follicle-associated epithelium and has been shown to be an important factor for intestinal colonization and persistence (Chassaing et al. 2011). The *pks* pathogenicity island is responsible for producing colibactin, a genotoxin that cause double-stranded DNA breaks and has been associated with CRC and tumor induction in a CRC mouse model (Arthur et al. 2012; Buc et al. 2013). In addition, other studies describing DAEC direct or indirect effects over epithelial cells may as well support the role of DAEC strains in IBD and CRC development. Infection of epithelial cells with DAEC C1845 strain induces loss of the adherent junction (AJ)-associated E-cadherin and cytokeratin 18 (Cane et al. 2010). In turn, loss of E-cadherin has been implicated in a cellular mechanism called epithelial to mesenchymal transition (EMT), a process heavily related to carcinoma progression in which epithelial cells are converted into motile cells (Thiery 2002). Hif-1α is overexpressed by the F1845-hDAF interaction and Hif-1α activates EMT mechanism, through the MAPK and PI3K signaling pathways (Cane et al. 2010). Hif-1α induces as well the expression of IL-8, vascular endothelial growth factor (VEGF) and Twist1 mRNA that has been also implicated in controlling the EMT mechanism (Cane et al. 2010; Lander et al. 2011). Moreover, infection of epithelial cells with Afa-1 and F1845-positive DAEC strains also induces upregulation of VEGF expression, through the activation of a Src protein kinase and Erk and Akt signaling pathways (Cane et al. 2007; Prorok-Hamon et al. 2014). On the other hand, abnormal expression of specific hCEACAMs, the other receptors of Afa/Dr adhesins, has been

related to cancer progression (Beauchemin and Arabzadeh 2013). Overall, DAEC infection of epithelial cells induces several pathways involved in angiogenesis and cell migration, essential steps for cancer development and growth (Beauchemin and Arabzadeh 2013).

9 Epidemiology

After DAEC strains were first described, it was important to establish their role in diarrheal illness, but this was controversial particularly because ingestion of DAEC isolates by adult volunteers did not result in illness (Scaletsky et al. 1984; Nataro et al. 1985; Mathewson et al. 1986; Tacket et al. 1990). Furthermore, some early descriptions indicated that DAEC strains were as frequently isolated from stool samples of diarrhea patients and controls (Levine et al. 1988; Gomes et al. 1989; Kang et al. 1995). Levine and colleagues, after conducting a cross-sectional cohort study of DEC strains prevalence in children, using molecular methods of detection, revealed both that the relative risk (RR) of DAEC infection increased with age and the difference in rate isolation between cases and controls was significant (Levine et al. 1993). Furthermore, in a study of children (<10 years of age) with diarrhea and controls, it was documented that DAEC strains were significantly more frequently isolated in the 2–6 years old group of children with diarrhea than in controls matched by age (Germani et al. 1996).

9.1 In the Americas

DEC history has always been closely related with Latin America and US scientists, since over the years several DEC epidemiological studies have been conducted and some pathogroups were first associated with diarrhea in children from Latin America, so DAEC is not the exception (Nataro and Kaper 1998). Although most studies that will be described here are from countries of the American continent, when relevant, studies from other regions will be discussed.

9.2 DAEC Age-Related Diarrheal Illness

A key study of DAEC illness associated with age was conducted in a low socioeconomic peri-urban community of Santiago, the capital of Chile (Levine et al. 1993). In this cross-sectional cohort study of 340 children from age birth to 47 months, it was observed that DAEC pathogenicity appeared to increase with age, while in the youngest age group (0–11 months), the RR of DAEC infection was 1.1; among subsequent age groups, the RR increased steadily reaching the highest RR value of

2.1 in children >48 months of age ($p=0.05$). Similarly, two studies of DEC molecular epidemiology, conducted in low socioeconomic level communities of Brazil, revealed only after age stratification a significant correlation between DAEC and diarrhea among children >12 months old and 2–3 years of age, from the Northeast and Espiritu Santo regions, respectively (Scaletsky et al. 2002b; Lozer et al. 2013). Similarly, in a study of DEC prevalence in children with and without diarrhea attending the emergency room (ER) of a Hospital in Espiritu Santo, Brazil, DAEC was significantly more often found in patients than controls ($P<0.05$), particularly among children >1 year of age ($P=0.01$) (Spano et al. 2008).

An age-related susceptibility study of infection of rotavirus and bacterial pathogens was conducted in peri-urban communities of Lima, Peru (Ochoa et al. 2009a). Of the 992 infants that were followed up during 13 months, the most common pathogens in diarrheal samples were DEC (31 %), *Campylobacter* (18.6), and rotavirus (17.2); DAEC was the third pathogen more frequently isolated from diarrhea samples among infants ≥6 months of age (Ochoa et al. 2009a). In accordance, an age-specific diarrhea association with DAEC was observed in this study.

A 4-year longitudinal study of bacterial pathogens causing diarrheal illness in children >5 years old was conducted in Mexico, and a total of 831 children were included, with most of them requiring treatment at the rehydration unit of a public tertiary hospital in Merida, Yucatan (Patzi-Vargas et al. 2015). DEC were the main bacterial agents identified among these children (28 %), followed by *Salmonella* (12 %) and *Shigella* (9 %), while *Campylobacter* was only identified in 5 % of samples. DAEC was the most prevalent pathogroup, the number of DAEC-diarrhea episodes tend to rise as children age increased, and DAEC was significantly associated with illness in children ≥6 months of age. Likewise, Spano and colleagues, after testing *E. coli* isolates with specific DNA probes designed to detect all six pathogroups, reported that DAEC was the most frequent pathogroup among children with diarrhea of a Pediatric Hospital in Espiritu Santo, Brazil (Spano et al. 2008).

9.3 DAEC Prevalence

Most recent reports of DAEC identification from *E. coli* strains isolated from stool samples of children and adults worldwide have employed PCR methods in addition to specific DAEC probes (Servin 2014). Together, these studies have clearly revealed the importance of selecting the proper *afa* or *daa* target genes, in order to establish a real DAEC prevalence among *E. coli* strains; this has been distinctly exemplified in studies conducted in countries of the American continent.

9.4 Children

Studies of DAEC prevalence in stools of children with diarrhea have shown diverse frequencies, from zero to less than 1 %, in investigations conducted in Costa Rica, Colombia and Brazil, and from 9 to 15 % in studies from Mexico and Peru,

respectively (Pérez et al. 2010; Rúgeles et al. 2010; Ochoa et al. 2011; Benevides-Matos et al. 2015; Patzi-Vargas et al. 2015). In the three studies where DAEC was negligibly identified, *daaE* was used as target gene for PCR-DAEC identification, whereas in the Peruvian and Mexican study, *daaD* and *afaC* genes were used, respectively. The *daaE* gene encodes for the F1845 fimbria, which has already been shown to be rarely present among *E. coli* strains isolated from stools of children and adults, worldwide (Campos et al. 1999; Rajendran et al. 2010; Mansan-Almeida et al. 2013; Shabana et al. 2013; Benevides-Matos et al. 2015). Furthermore, *afaE*/*daaE* genes are the less conserved among Afa/Dr adhesin operons, whereas both *afaCD*/*daaCD* genes are highly conserved (Servin 2014). Of note, from the 3100 *E. coli* strains isolated in the Mexican study, only 2 (0.06 %) strains simultaneously harbored genes encoding for *afaC* and EAEC genes (Patzi-Vargas et al. 2015). The Peruvian study that analyzed *E. coli* isolates of children stools collected from a variety of clinical settings and regions clearly revealed that DAEC prevalence was dependent on these parameters (Ochoa et al. 2011). DAEC overall prevalence was 4.8 % in isolates from children with diarrhea, but it was as low as 1.7 % in HIV children, and as high as 15 % in hospitalized children with acute diarrhea.

In the United States, where it has been shown that most childhood diarrheal diseases are caused by pathogens not recognized in routine clinical testing, few studies have been conducted searching for DEC. In a Cincinnati Hospital, enteric pathogens, including DEC, were sought out in 1327 children ≤5 years old (ER patients and inpatients) with diarrhea and in 555 matched control children (Cohen et al. 2005). DEC were isolated significantly more often from patients attending the ER than from control subjects; among the 563 ER patients, DAEC (13 %)>typical EAEC (9 %)>aEPEC (6 %) were more frequently identified (Cohen et al. 2005). A similar study was conducted at a Tennessee hospital; of the 206 children (<12 years of age) with diarrhea and/or vomiting, only 12 (5.8 %) patients were positive for DEC, 8 for tEAEC and 4 for aEPEC; noteworthy, DAEC was not identified using the daaE primers (Foster et al. 2015). Together, these results suggest that in the US DEC may be an important unrecognized cause of diarrhea in children. Thus, health authorities should encourage more studies of DEC prevalence in children with diarrhea, and DAEC should be characterized by PCR techniques using primers for *afaC* or *daaD* genes.

9.5 Adults

In general, studies of diarrhea illness among adults are scarce worldwide, with the exception of diarrhea studies among adult travelers from industrialized countries visiting less developed areas of the world, known as travelers' diarrhea (TD). Stool specimens from 350 Spanish travelers with diarrhea, some returning from Central and South America, were examined for intestinal pathogens (Vargas et al. 1998). DEC identified by PCR techniques were the most prevalent pathogens among TD patients (107, 30.5 %) and DAEC (9.1 %) was the third most prevalent pathogroup, just after ETEC (15.7 %) and EAEC (13.4 %). In a TD study, *E. coli* strains were recovered from 162 stools of 54, 39, and 69 patients returning from Guatemala,

Mexico, and India, respectively. DAEC strains carrying *afa/dra* genes were implicated in 11 % of the cases and it was found in approximately equal frequency (~11 %) in each study site (Meraz et al. 2008). Mansan-Almeida and colleagues collected *E. coli* strains from Brazilian children and adults with and without diarrhea, DAEC strains harboring *afa*/*dra* adhesin genes were similarly detected in children cases and controls, 16.5 % and 19.6 %, respectively. DAEC was significantly ($p < 0.01$) more often recovered from adults with diarrhea (18.8 %) than from controls (4.2 %) (Mansan-Almeida et al. 2013). Further characterization of DAEC isolates for the presence and expression of virulence genes revealed a significant association with diarrhea ($p < 0.05$) of Sat and curli fimbriae, among children and adult strains, respectively; suggesting that DAEC strains causing diarrhea in children and adults constitute two different populations (Mansan-Almeida et al. 2013).

9.6 HIV

HIV studies conducted among children and adults with diarrhea confirmed that DAEC strains constitute two different populations. Two independent studies were conducted among Peruvian HIV-positive adults and children with and without diarrhea. In both studies, DEC was sought out by use of a melting curve analysis and RT-PCR during the same period of time, *daaD* was the target gene for DAEC identification (Garcia et al. 2010; Medina et al. 2010). In the adult study, 184 subjects were included (mean age 35.6 years): HIV-positive patients and controls, with and without diarrhea. DEC, particularly ETEC, was more common among patients with diarrhea (42 % vs. 20 %, $P < 0.05$), while DAEC was only present in HIV-positive patients with diarrhea (10.1 %) (Garcia et al. 2010). In contrast, in the children study, DAEC was not identified, even after children were stratified by age, in any of the 140 HIV-positive children with and without diarrhea (Medina et al. 2010). So, it will be important to determine if DAEC strains isolated from HIV adult patients with diarrhea harbor or express curli.

9.7 Antibiotics Resistance

Most cases of diarrhea exhibit mild symptoms and resolve quickly without antibiotic therapy. However, patients should be treated if they are debilitated, particularly with malignancy, immunosuppressed, have chronic disease, such as diabetes, or are extremely young or old. Treatment is also advised for those cases who relapse and for those with severe or prolonged symptoms (Casburn-Jones and Farthing 2004). Currently, quinolone antibiotics are the treatment of choice. Nonetheless, fluoroquinolones are not approved for pregnant women and children, where azithromycin is also a good option (Casburn-Jones and Farthing 2004).

Although antimicrobial therapies are successful to ameliorate the course of illness with many intestinal pathogens, the emergence of antibiotic-resistant strains has created the necessity of analyzing these strains to develop effective treatments. Different studies have reported that most DAEC strains isolated either from children with diarrhea or asymptomatic children, were resistant to several antibiotics such as: ampicillin, cotrimoxazole, tetracycline, nalidixic acid, chloramphenicol, ciprofloxacin, kanamycin, ofloxacin, sulfonamide, cephalotin, and streptomycin, including an important proportion of multidrug-resistant strains (resistance to three or more antibiotics), drugs commonly used to treat pediatric diarrhea (Le Bouguenec et al. 1993; Souza et al. 2009; Ochoa et al. 2009b; Patzi-Vargas et al. 2013). On the other hand, some authors have reported susceptibility to ceftazidime, gentamicin, lomefloxacin, trimethoprim-sulfamethoxazole, and azithromycin (Lopes et al. 2005; Patzi-Vargas et al. 2013).

Acknowledgments CONACYT scholarship 368026 to M M-S. We thank Diego Meza Segura for realization of drawings

References

Anderson KL, Billington J, Pettigrew D, Cota E, Simpson P, Roversi P, Chen HA, Urvil P, Du Merle L, Barlow PN, Medof ME, Smith RAG, Nowicki B, Le Bouguénec C, Lea SM, Matthews S (2004) An atomic resolution model for assembly, architecture, and function of the Dr adhesins. Mol Cell 15:647–657

Andoh A, Fujiyama Y, Sakumoto H, Bamba T (1996) Interleukin 4 acts as an inducer of decay-accelerating factor gene expression in human intestinal epithelial cells. Gastroenterology 111:911–918

Andoh A, Fujiyama Y, Sumiyoshi K, Sakumoto H, Okabej H, Departments TB (1997) Tumour necrosis factor-α up-regulates decay-accelerating factor gene expression in human intestinal epithelial cells. Immunology 90:358–363

Arikawa K, Nishikawa Y (2010) Interleukin-8 induction due to diffusely adherent *Escherichia coli* possessing Afa/Dr genes depends on flagella and epithelial Toll-like receptor 5. Microbiol Immunol 54:491–501

Arikawa K, Meraz IM, Nishikawa Y, Ogasawara J, Hase A (2005) Interleukin-8 secretion by epithelial cells infected with diffusely adherent *Escherichia coli* possessing Afa adhesin-coding genes. Microbiol Immunol 49:493–503

Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C (2012) Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338:120–123

Baqui AH, Sack RB, Black RE, Haider K, Hossain A, Alim AR, Yunus M, Chowdhury HR, Siddique AK (1992) Enteropathogens associated with acute and persistent diarrhea in Bangladeshi children less than 5 years of age. J Infect Dis 166:792–796

Barnhart MM, Chapman MR (2006) Curli biogenesis and function. Annu Rev Microbiol 60:131–147

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285:727–729

Beauchemin N, Arabzadeh A (2013) Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. Cancer Metastasis Rev 32:643–671

Beinke C, Laarmann S, Wachter C, Karch H, Greune L, Schmidt MA (1998) Diffusely adhering *Escherichia coli* strains induce attaching and effacing phenotypes and secrete homologs of Esp proteins. Infect Immun 66:528–539

Benevides-Matos N, Pieri FA, Penatti M, Orlandi PP (2015) Adherence and virulence genes of *Escherichia coli* from children diarrhoea in the Brazilian Amazon. Braz J Microbiol 46:131–137

Berger CN, Billker O, Meyer TF, Servin AL, Kansau I (2004) Differential recognition of members of the carcinoembryonic antigen family by Afa/Dr adhesins of diffusely adhering *Escherichia coli* (Afa/Dr DAEC). Mol Microbiol 52:963–983

Bernet-Camard MF, Coconnier MH, Hudault S, Servin AL (1996a) Differentiation-associated antimicrobial functions in human colon adenocarcinoma cell lines. Exp Cell Res 226:80–89

Bernet-Camard MF, Coconnier MH, Hudault S, Servin AL (1996b) Pathogenicity of the diffusely adhering strain *Escherichia coli* C1845: F1845 adhesin-decay accelerating factor interaction, brush border microvillus injury, and actin disassembly in cultured human intestinal epithelial cells. Infect Immun 64:1918–1928

Bétis F, Brest P, Hofman V, Guignot J, Bernet-Camard MF, Rossi B, Servin A, Hofman P (2003a) The Afa/Dr adhesins of diffusely adhering *Escherichia coli* stimulate interleukin-8 secretion, activate mitogen-activated protein kinases, and promote polymorphonuclear transepithelial migration in T84 polarized epithelial cells. Infect Immun 71:1068–1074

Bétis F, Brest P, Hofman V, Guignot J, Kansau I, Rossi B, Servin A, Hofman P (2003b) Afa/Dr diffusely adhering *Escherichia coli* infection in T84 cell monolayers induces increased neutrophil transepithelial migration, which in turn promotes cytokine-dependent upregulation of decay-accelerating factor (CD55), the receptor for Afa/Dr adhesin. Infect Immun 71:1774–1783

Bilge SS, Clausen CR, Lau W, Moseley SL (1989) Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated *Escherichia coli* to HEp-2 cells. J Bacteriol 171:4281–4289

Bilge SS, Apostol JM Jr, Fullner KJ, Moseley SL (1993) Transcriptional organization of the F1845 fimbrial adhesin determinant of *Escherichia coli*. Mol Microbiol 7:993–1006

Blanc-Potard AB, Tinsley C, Scaletsky I, Le Bouguenec C, Guignot J, Servin AL, Nassif X, Bernet-Camard M-F (2002) Representational difference analysis between Afa/Dr diffusely adhering *Escherichia coli* and nonpathogenic *E. coli* K-12. Infect Immun 70:5503–5511

Brest P, Bétis F, Cuburu N, Selva E, Herrant M, Servin A, Auberger P, Hofman P (2004) Increased rate of apoptosis and diminished phagocytic ability of human neutrophils infected with Afa/Dr diffusely adhering *Escherichia coli* strains. Infect Immun 72:5741–5749

Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol 198:773–783

Buc E, Dubois D, Sauvanet P, Raisch J, Delmas J, Darfeuille-Michaud A, Pezet D, Bonnet R (2013) High prevalence of mucosa-associated *E. coli* producing cyclomodulin and genotoxin in colon cancer. PLoS One 8, e56964

Campos LC, Vieira MA, Trabulsi LR, da Silva LA, Monteiro-Neto V, Gomes TA (1999) Diffusely adhering *Escherichia coli* (DAEC) strains of fecal origin rarely express F1845 adhesin. Microbiol Immunol 43:167–170

Cane G, Moal VL-L, Pagès G, Servin AL, Hofman P, Vouret-Craviari V (2007) Up-regulation of intestinal vascular endothelial growth factor by Afa/Dr diffusely adhering *Escherichia coli*. PLoS One 2, e1359

Cane G, Ginouvès A, Marchetti S, Buscà R, Pouysségur J, Berra E, Hofman P, Vouret-Craviari V (2010) HIF-1α mediates the induction of IL-8 and VEGF expression on infection with Afa/Dr diffusely adhering *E. coli* and promotes EMT-like behaviour. Cell Microbiol 12:640–653

Carnoy C, Moseley SL (1997) Mutational analysis of receptor binding mediated by the Dr family of *Escherichia coli* adhesins. Mol Microbiol 23:365–379

Casburn-Jones A, Farthing M (2004) Management of infectious diarrhoea. Gut 53:296–305

Chassaing B, Rolhion N, de Vallée A, Salim SY, Prorok-Hamon M, Neut C, Campbell BJ, Söderholm JD, Hugot J, Colombel J-F, Darfeuille-Michaud A (2011) Crohn disease-associated adherent-invasive *E. coli* bacteria target mouse and human Peyer's patches via long polar fimbriae. J Clin Invest 121:966–975

Cohen MB, Nataro JP, Bernstein DI, Hawkins J, Roberts N, Staat MA (2005) Prevalence of diarrheagenic *Escherichia coli* in acute childhood enteritis: a prospective controlled study. J Pediatr 146:54–61

Cookson ST, Nataro JP (1996) Characterization of HEp-2 cell projection formation induced by diffusely adherent *Escherichia coli*. Microb Pathog 21:421–434

Cota E, Jones C, Simpson P, Altroff H, Anderson KL, Du Merle L, Guignot J, Servin A, Le Bouguénec C, Mardon H, Matthews S (2006) The solution structure of the invasive tip complex from Afa/Dr fibrils. Mol Microbiol 62:356–366

Das M, Hart-Van Tassell A, Urvil PT, Lea S, Pettigrew D, Anderson KL, Samet A, Kur J, Matthews S, Nowicki S, Popov V, Goluszko P, Nowicki BJ (2005) Hydrophilic domain II of *Escherichia coli* Dr fimbriae facilitates cell invasion. Infect Immun 73:6119–6126

Dautin N (2010) Serine protease autotransporters of *Enterobacteriaceae* (SPATEs): biogenesis and function. Toxins (Basel) 2:1179–1206

Duguid J, Smith I, Dempster G, Edmunds P (1955) Non-flagellar filamentous appendages (fimbriae) and haemagglutinating activity in *Bacterium coli*. J Pathol Bacteriol 70:335–348

Escobar-Páramo P, Clermont O, Blanc-Potard AB, Bui H, Le Bouguénec C, Denamur E (2004) A specific genetic background is required for acquisition and expression of virulence factors in *Escherichia coli*. Mol Biol Evol 21:1085–1094

Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ (2015) Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol 13:269–284

Foster MA, Iqbal J, Zhang C, Mchenry R, Cleveland BE, Romero-Herazo Y, Fonnesbeck C, Payne DC, Chappell JD, Halasa N, Gómez-Duarte OG (2015) Enteropathogenic and enteroaggregative *E. coli* in stools of children with acute gastroenteritis in Davidson County, Tennessee. Diagn Microbiol Infect Dis 83:319–324

Garcia MI, Labigne A, Le Bouguenec C (1994) Nucleotide sequence of the afimbrial-adhesin-encoding *afa-3* gene cluster and its translocation via flanking IS1 insertion sequences. J Bacteriol 176:7601–7613

Garcia MI, Jouve M, Nataro JP, Gounon P, Le Bouguénec C (2000) Characterization of the AfaD-like family of invasins encoded by pathogenic *Escherichia coli* associated with intestinal and extra-intestinal infections. FEBS Lett 479:111–117

Garcia C, Chincha O, Leon M, Iglesias D, Barletta F, Mercado E, Ochoa T (2010) Short report: high frequency of diarrheagenic *Escherichia coli* in human immunodeficiency virus (HIV) patients with and without diarrhea in Lima, Peru. Am J Trop Med Hyg 82:1118–1120

Germani Y, Bégaud E, Duval P, Le Bouguénec C (1996) Prevalence of enteropathogenic, enteroaggregative, and diffusely adherent *Escherichia coli* among isolates from children with diarrhea in new Caledonia. J Infect Dis 174:1124–1126

Goldhar J (1996) Nonfimbrial adhesins of *Escherichia coli*. Adv Exp Med Biol 408:63–72

Goluszko P, Popov V, Selvarangan R, Nowicki S, Pham T, Nowicki BJ (1997) Dr fimbriae operon of uropathogenic *Escherichia coli* mediate microtubule-dependent invasion to the HeLa epithelial cell line. J Infect Dis 176:158–167

Gomes TAT, Blake PA, Trabulsi LR (1989) Prevalence of *Escherichia coli* strains with localized, diffuse, and aggregative adherence to HeLa cells in infants with diarrhea and matched controls. J Clin Microbiol 27:266–269

Guignot J, Peiffer I, Bernet-Camard MF, Lublin DM, Carnoy C, Moseley SL, Servin AL (2000) Recruitment of CD55 and CD66e brush border-associated glycosylphosphatidylinositol-anchored proteins by members of the Afa/Dr diffusely adhering family of *Escherichia coli* that infect the human polarized intestinal Caco-2/TC7 cells. Infect Immun 68:3554–3563

Guignot J, Bernet-Camard MF, Poüs C, Plançon L, Le Bouguenec C, Servin AL (2001) Polarized entry of uropathogenic Afa/Dr diffusely adhering *Escherichia coli* strain IH11128 into human epithelial cells: evidence for $\alpha_5\beta_1$ integrin recognition and subsequent internalization through a pathway involving caveolae and dynamic unstable microtubules. Infect Immun 69:1856–1868

Guignot J, Chaplais C, Coconnier-Polter MH, Servin AL (2007) The secreted autotransporter toxin, Sat, functions as a virulence factor in Afa/Dr diffusely adhering *Escherichia coli* by promoting lesions in tight junction of polarized epithelial cells. Cell Microbiol 9:204–221

Guignot J, Hudault S, Kansau I, Chau I, Servin AL (2009) Human decay-accelerating factor and CEACAM receptor-mediated internalization and intracellular lifestyle of Afa/Dr diffusely adhering *Escherichia coli* in epithelial cells. Infect Immun 77:517–531

Hasan RJ, Pawelczyk E, Urvil PT, Venkatarajan MS, Goluszko P, Kur J, Selvarangan R, Nowicki S, Braun WA, Nowicki BJ (2002) Structure-function analysis of decay-accelerating factor: Identification of residues important for binding of the *Escherichia coli* Dr adhesin and complement regulation. Infect Immun 70:4485–4493

Hernandes RT, Elias WP, Vieira MAM, Gomes TA (2009) An overview of atypical enteropathogenic *Escherichia coli*. FEMS Microbiol Lett 297:137–149

Hudault S, Spiller OB, Morgan BP, Servin AL (2004) Human diffusely adhering *Escherichia coli* expressing Afa/Dr adhesins that use human CD55 (decay-accelerating factor) as a receptor does not bind the rodent and pig analogues of CD55. Infect Immun 72:4859–4863

Jouve M, Garcia MI, Courcoux P, Labigne A, Gounon P, Le Bouguénec C (1997) Adhesion to and invasion of HeLa cells by pathogenic *Escherichia coli* carrying the *afa-3* gene cluster are mediated by the AfaE and AfaD proteins, respectively. Infect Immun 65:4082–4089

Kang G, Mathan MM, Mathan VI (1995) Evaluation of a simplified HEp-2 cell adherence assay for *Escherichia coli* isolated from south Indian children with acute diarrhea and controls. J Clin Microbiol 33:2204–2205

Kansau I, Berger CN, Hospital M, Amsellem R, Nicolas V, Servin AL, Bernet-Camard MF (2004) Zipper-like internalization of Dr-positive *Escherichia coli* by epithelial cells is preceded by an adhesin-induced mobilization of raft-associated molecules in the initial step of adhesion. Infect Immun 72:3733–3742

Keller R, Ordoñez JG, De Oliveira RR, Trabulsi LR, Baldwin TJ, Knutton S (2002) Afa, a diffuse adherence fibrillar adhesin associated with enteropathogenic *Escherichia coli*. Infect Immun 70:2681–2689

Kerneis S, Bilge SS, Fourel V, Chauviere G, Coconnier MH, Servin AL (1991) Use of purified F1845 fimbrial adhesin to study localization and expression of receptors for diffusely adhering *Escherichia coli* during enterocytic differentiation of human colon carcinoma cell lines HT-29 and Caco-2 in culture. Infect Immun 59:4013–4018

Korotkova N, Cota E, Lebedin Y, Monpouet S, Guignot J, Servin AL, Matthews S, Moseley SL (2006) A subfamily of Dr adhesins of *Escherichia coli* bind independently to decay-accelerating factor and the N-domain of carcinoembryonic antigen. J Biol Chem 281:29120–29130

Korotkova N, Yarova-Yarovaya Y, Tchesnokova V, Yazvenko N, Carl MA, Stapleton AE, Moseley SL (2008) *Escherichia coli* DraE adhesin-associated bacterial internalization by epithelial cells is promoted independently by decay-accelerating factor and carcinoembryonic antigen-related cell adhesion molecule binding and does not require the DraD invasin. Infect Immun 76:3869–3880

Kuespert K, Pils S, Hauck CR (2006) CEACAMs: their role in physiology and pathophysiology. Curr Opin Cell Biol 18:565–571

Kyaw CM, De Araujo CR, Lima MR, Gondim EGS, Brígido MM, Giugliano LG (2003) Evidence for the presence of a type III secretion system in diffusely adhering *Escherichia coli* (DAEC). Infect Genet Evol 3:111–117

Labigne-Roussel A, Falkow S (1988) Distribution and degree of heterogeneity of the afimbrial-adhesin-encoding operon (*afa*) among uropathogenic *Escherichia coli* isolates. Infect Immun 56:640–648

Labinge-Roussel AF, Lark D, Schoolnik G, Falkow S (1984) Cloning and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemagglutination from a pyelonephritic *Escherichia coli* strain. Infect Immun 46:251–259

Lalioui L, Jouve M, Gounon P, Le Bouguenec C (1999) Molecular cloning and characterization of the *afa-7* and *afa-8* gene clusters encoding afimbrial adhesins in *Escherichia coli* strains associated with diarrhea or septicemia in calves. Infect Immun 67:5048–5059

Lander R, Nordin K, Labonne C (2011) The F-box protein Ppa is a common regulator of core EMT factors Twist, Snail, Slug, and Sip1. J Cell Biol 194:17–25

Le Bouguenec C, Garcia MI, Ouin V, Desperrier J-M, Gounon P, Labigne A (1993) Characterisation of plasmid-borne *afa-3* gene cluster encoding afimbrial adhesins expressed by *Escherichia coli* strains associated with intestinal or urinary tract infections. Infect Immun 61:5106–5114

Levine MM, Prado V, Robins-Browne R, Lior H, Kaper JB, Moseley SL, Gicquelais K, Nataro JP, Vial P, Tall B (1988) Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. J Infect Dis 158:224–228

Levine MM, Ferreccio C, Prado V, Cayazzo M, Abrego P, Martinez J, Maggi L, Baldini MM, Martin W, Maneval D, Kay B, Guers L, Lior H, Wasserman SS, Nataro JP (1993) Epidemiologic studies of *Escherichia coli* diarrheal infections in a low socioeconomic level peri-urban community in Santiago, Chile. Am J Epidemiol 138:849–869

Lopes LM, Fabbricotti SH, Ferreira AJP, Kato MAMF, Michalski J, Scaletsky ICA (2005) Heterogeneity among strains of diffusely adherent *Escherichia coli* isolated in Brazil. J Clin Microbiol 43:1968–1972

López-Saucedo C, Cerna JF, Villegas-sepulveda N, Thompson R, Velazquez FR, Torres J, Tarr PI, Estrada-García T (2003) Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg Infect Dis 9:127–131

Lozer D, Souza T, Monfardini MV, Vicentini F, Kitagawa SS, Scaletsky ICA, Spano LC (2013) Genotypic and phenotypic analysis of diarrheagenic *Escherichia coli* strains isolated from Brazilian children living in low socioeconomic level communities. BMC Infect Dis 13:418

Lublin DM (2005) Review: Cromer and DAF: role in health and disease. Immunohematology 21:39–47

Mansan-Almeida R, Pereira AL, Giugliano LG (2013) Diffusely adherent *Escherichia coli* strains isolated from children and adults constitute two different populations. BMC Microbiol 13:22

Marin-Esteban V, Turbica I, Dufour G, Semiramoth N, Gleizes A, Gorges R, Beau I, Servin AL, Moal VL, Sandré C, Chollet-Martin S (2012) Afa/Dr diffusely adhering *Escherichia coli* strain C1845 induces neutrophil extracellular traps that kill bacteria and damage human enterocyte-like cells. Infect Immun 80:1891–1899

Mathewson JJ, Johnson PC, Dupont HL, Satterwhite TK, Winsor DK (1986) Pathogenicity of enteroadherent *Escherichia coli* in adult volunteers. J Infect Dis 154:524–527

Medina AM, Rivera FP, Romero LM, Kolevic LA, Castillo ME, Verne E, Hernandez R, Mayor YE, Barletta F, Mercado E, Ochoa TJ (2010) Diarrheagenic *Escherichia coli* in human immunodeficiency virus (HIV) pediatric patients in Lima, Perú. Am J Trop Med Hyg 83:158–163

Meraz IM, Jiang ZD, Ericsson CD, Bourgeois AL, Steffen R, Taylor DN, Hernandez N, DuPont HL (2008) Enterotoxigenic *Escherichia coli* and diffusely adherent *E coli* as likely causes of a proportion of pathogen-negative travelers' diarrhea-A PCR-based study. J Travel Med 15:412–418

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201

Nataro JP, Scaletsky ICA, Kaper JB, Levine MM, Trabulsi LR (1985) Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. Infect Immun 48:378–383

Nowicki B, Hart A, Coyne KE, Lublin DM, Nowicki S (1993) Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by *Escherichia coli* recombinant Dr adhesin in a model of a cell-cell interaction. J Exp Med 178:2115–2121

Nowicki B, Selvarangan R, Nowicki S (2001) Family of *Escherichia coli* Dr adhesins: decay-accelerating factor receptor recognition and invasiveness. J Infect Dis 183(suppl):S24–S27

Ochoa TJ, Ecker L, Barletta F, Mispireta ML, Gil AI, Contreras C, Molina M, Amemiya I, Verastegui H, Hall ER, Cleary TG, Lanata CF (2009a) Age-related susceptibility to infection with diarrheagenic *Escherichia coli* among infants from Periurban areas in Lima, Peru. Clin Infect Dis 49:1694–1702

Ochoa TJ, Ruiz J, Molina M, Del Valle LJ, Vargas M, Gil AI, Ecker L, Barletta F, Hall E, Cleary TG, Lanata CF (2009b) High frequency of antimicrobial drug resistance of diarrheagenic *Escherichia coli* in infants in Peru. Am J Trop Med Hyg 81:296–301

Ochoa TJ, Mercado EH, Durand D, Rivera FP, Mosquito S, Contreras C, Riveros M, Lluque A, Barletta F, Prada A, Ruiz J (2011) Frecuencia y patotipos de *Escherichia coli* diarrogénica en niños peruanos con y sin diarrea. Rev Peru Med Exp Salud Publica 28:13–20

Patzi-Vargas S, Zaidi M, Bernal-Reynaga R, León-Cen M, Michel A, Estrada-Garcia T (2013) Persistent bloody diarrhoea without fever associated with diffusely adherent *Escherichia coli* in a young child. J Med Microbiol 62:1907–1910

Patzi-Vargas S, Zaidi MB, Perez-Martinez I, León-Cen M, Michel-Ayala A, Chaussabel D, Estrada-Garcia T (2015) Diarrheagenic *Escherichia coli* carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis 9:1–18

Peiffer I, Blanc-Potard A-B, Bernet-Camard M-F, Guignot J, Barbat A, Servin AL (2000a) Afa/Dr diffusely adhering *Escherichia coli* C1845 infection promotes selective injuries in the junctional domain of polarized human intestinal Caco-2/TC7 cells. Infect Immun 68:3431–3442

Peiffer I, Guignot J, Barbat A, Carnoy C, Moseley SL, Nowicki BJ, Servin AL, Bernet-Camard MF (2000b) Structural and functional lesions in brush border of human polarized intestinal Caco-2/TC7 cells infected by members of the Afa/Dr diffusely adhering family of *Escherichia coli*. Infect Immun 68:5979–5990

Peiffer I, Bernet-Camard MF, Rousset M, Servin AL (2001) Impairments in enzyme activity and biosynthesis of brush border-associated hydrolases in human intestinal Caco-2/TC7 cells infected by members of the Afa/Dr family of diffusely adhering *Escherichia coli*. Cell Microbiol 3:341–357

Pérez C, Gómez-Duarte OG, Arias ML (2010) Diarrheagenic *Escherichia coli* in children from Costa Rica. Am J Trop Med Hyg 83:292–297

Plançon L, du Merle L, Le Friec S, Gounon P, Jouve M, Guignot J, Servin A, Le Bouguénec C (2003) Recognition of the cellular β1-chain integrin by the bacterial AfaD invasin is implicated in the internalization of *afa*-expressing pathogenic *Escherichia coli* strains. Cell Microbiol 5:681–693

Prorok-Hamon M, Friswell MK, Alswied A, Roberts CL, Song F, Flanagan PK, Knight P, Codling C, Marchesi JR, Winstanley C, Hall N, Rhodes JM, Campbell BJ (2014) Colonic mucosa-associated diffusely adherent *afaC+ Escherichia coli* expressing *lpfA* and *pks* are increased in inflammatory bowel disease and colon cancer. Gut 63:761–770

Queval CJ, Nicolas V, Beau I (2011) Role of Src kinases in mobilization of glycosylphosphatidylinositol-anchored decay-accelerating factor by Dr fimbria-positive adhering bacteria. Infect Immun 79:2519–2534

Rajendran P, Ajjampur SSR, Chidambaram D, Chandrabose G, Thangaraj B, Sarkar R, Samuel P, Rajan DP, Kang G (2010) Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India. Diagn Microbiol Infect Dis 68:117–122

Rúgeles LC, Bai J, Martínez AJ, Vanegas MC, Gómez-Duarte OG (2010) Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. Int J Food Microbiol 138:282–286

Scaletsky ICA, Silva MLM, Trabulsi LR (1984) Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect Immun 45:534–536

Scaletsky ICA, Fabbricotti SH, Aranda KR, Morais MB, Fagundes-Neto U (2002a) Comparison of DNA hybridization and PCR assays for detection of putative pathogenic enteroadherent *Escherichia coli*. J Clin Microbiol 40:1254–1258

Scaletsky ICA, Fabbricotti SH, Carvalho RLB, Nunes CR, Maranhão HS, Morais MB, Fagundes-Neto U (2002b) Diffusely adherent Escherichia coli as a cause of acute diarrhea in young children in Northeast Brazil: a case-control study. J Clin Microbiol 40:645–648

Scaletsky ICA, Fabbricotti SH, Silva SOC, Morais MB, Fagundes-Neto U (2002c) HEp-2-adherent *Escherichia coli* strains associated with acute infantile diarrhea, São Paulo, Brazil. Emerg Infect Dis 8:855–858

Selvarangan R, Goluszko P, Popov V, Singhal J, Pham T, Lublin DM, Nowicki S, Nowicki B (2000) Role of decay-accelerating factor domains and anchorage in internalization of Dr-fimbriated *Escherichia coli*. Infect Immun 68:1391–1399

Sémiramoth N, Gleizes A, Turbica I, Sandré C, Gorges R, Kansau I, Servin A, Chollet-Martin S (2009) *Escherichia coli* type 1 pili trigger late IL-8 production by neutrophil-like differentiated PLB-985 cells through a Src family kinase- and MAPK-dependent mechanism. J Leukoc Biol 85:310–321

Sémiramoth N, Gleizes A, Turbica I, Sandré C, Marin-Esteban V, Gorges R, Servin A, Chollet-Martin S (2010) Afa/Dr-expressing, diffusely adhering *Escherichia coli* strain C1845 triggers F1845 fimbria-dependent phosphatidylserine externalization on neutrophil-like differentiated PLB-985 cells through an apoptosis-independent mechanism. Infect Immun 78:2974–2983

Servin AL (2014) Pathogenesis of human diffusely adhering *Escherichia coli* expressing Afa/Dr adhesins (Afa/Dr DAEC): current insights and future challenges. Clin Microbiol Rev 27:823–869

Shabana II, Zaraket H, Suzuki H (2013) Molecular studies on diarrhea-associated *Escherichia coli* isolated from humans and animals in Egypt. Vet Microbiol 167:532–539

Shifrin D, McConnell R, Nambiar R, Higginbotham J, Coffey R, Tyska M (2013) Enterocyte microvillus-derived vesicles detoxify bacterial products and regulate epithelial-microbial interactions. Curr Biol 22:627–631

Smith HR, Scotland SM, Willshaw GA, Rowe B, Cravioto A, Eslava C (1994) Isolates of *Escherichia coli* 044:H18 of diverse origin are enteroaggregative. J Infect Dis 170:1610–1613

Snelling AM, Macfarlane-Smith LR, Fletcher JN, Okeke IN (2009) The commonly-used DNA probe for diffusely-adherent *Escherichia coli* cross-reacts with a subset of enteroaggregative *E. coli*. BMC Microbiol 9:269

Souza TB, Morais MB, Tahan S, Melli LCFL, Rodrigues MSC, Scaletsky ICA (2009) High prevalence of antimicrobial drug-resistant diarrheagenic *Escherichia coli* in asymptomatic children living in an urban slum. J Infect 59:247–251

Spano LC, Sadovsky ADI, Segui PN, Saick KW, Kitagawa SMS, Pereira FEL, Fagundes-Neto U, Scaletsky ICA (2008) Age-specific prevalence of diffusely adherent *Escherichia coli* in Brazilian children with acute diarrhoea. J Med Microbiol 57:359–363

Tacket CO, Moseley SL, Kay B, Losonsky G, Levine MM (1990) Challenge studies in volunteers using *Escherichia coli* strains with diffuse adherence to HEp-2 cells. J Infect Dis 162:550–552

Taddei CR, Moreno ACR, Fernandes Filho A, Montemor LPG, Martinez MB (2003) Prevalence of secreted autotransporter toxin gene among diffusely adhering *Escherichia coli* isolated from stools of children. FEMS Microbiol Lett 227:249–253

Taddei CR, Fasano A, Ferreira AJP, Trabulsi LR, Martinez MB (2005) Secreted autotransporter toxin produced by a diffusely adhering *Escherichia coli* strain causes intestinal damage in animal model assays. FEMS Microbiol Lett 250:263–269

Tanimoto Y, Arikawa K, Nishikawa Y (2013) Effect of diffusely adherent *Escherichia coli* strains isolated from diarrhoeal patients and healthy carriers on IL-8 secretion and tight junction barrier integrity of Caco-2 cells. Vet Immunol Immunopathol 152:183–188

Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454

Tieng V, Le Bouguénec C, du Merle L, Bertheau P, Desreumaux P, Janin A, Charron D, Toubert A (2002) Binding of *Escherichia coli* adhesin AfaE to CD55 triggers cell-surface expression of the MHC class I-related molecule MICA. Proc Natl Acad Sci U S A 99:2977–2982

Tükel Ç, Wilson RP, Nishimori JH, Pezeshki M, Chromy BA, Bäumler AJ (2009) Responses to amyloids of microbial and host origin are mediated through Toll-like receptor 2. Cell Host Microbe 6:45–53

Vargas M, Gascón J, Gallardo F, Jimenez De Anta MT, Vila J (1998) Prevalence of diarrheagenic *Escherichia coli* strains detected by PCR in patients with travelers' diarrhea. Clin Microbiol Infect 4:682–688

Yamamoto T, Koyama Y, Matsumoto M, Sonoda E, Nakayama S, Uchimura M, Paveenkittiporn W, Tamura K, Yokota T, Echeverria P (1992) Localized, aggregative, and diffuse adherence to HeLa cells, plastic, and human small intestines by *Escherichia coli* isolated from patients with diarrhea. J Infect Dis 166:1295–1310

Yamamoto T, Kaneko M, Changchawalit S, Serichantalergs O, Ijuin S, Echeverria P (1994) Actin accumulation associated with clustered and localized adherence in *Escherichia coli* isolated from patients with diarrhea. Infect Immun 62:2917–2929

Zav'yalov V, Zavialov A, Zav'Yalova G, Korpela T (2010) Adhesive organelles of Gram-negative pathogens assembled with the classical chaperone/usher machinery: structure and function from a clinical standpoint. FEMS Microbiol Rev 34:317–378

Chapter 7
Escherichia coli in Animals

Analía I. Etcheverría, Paula M.A. Lucchesi, Alejandra Krüger, Adriana B. Bentancor, and Nora L. Padola

Summary Escherichia coli is the most widely studied bacterium over the world. It is well-known that E. coli is the predominant non-pathogenic microbiota of warm blood species; however, some strains have developed the ability to cause severe diseases. Taking into account the diversity in American countries, this chapter examines the complex situation of puzzling intestinal pathogenic E. coli, also called diarrheagenic, (enteropathogenic E. coli, Shiga toxin-producing E. coli, enterotoxigenic E. coli, enteroaggregative E. coli, enteroinvasive E. coli, diffusely adherent E. coli), and extra-intestinal E. coli (uropathogenic E. coli, neonatal meningitis-associated E. coli, avian pathogenic E. coli, sepsis-associated E. coli, mammary pathogenic E. coli, endometrial pathogenic E. coli, and necrotoxigenic E. coli) in animals. In addition to E. coli-associated animal diseases, the role of carriers and reservoirs is presented, including the last regional references from synanthropic and wild animals. Findings of the last 5 years are discussed and data of the eco-epidemiology of E. coli is also included. Considering the concept of One Health, which recognizes that health of humans is connected to health of animals and the environment, the strategies to diminish illness in human population cannot exclude control and vigilance of pathogenic strains in animals. However, in E. coli control,

A.I. Etcheverría • P.M.A. Lucchesi (✉) • A. Krüger • N.L. Padola
CIVETAN, UNCPBA, CICPBA, CONICET, Facultad de Ciencias Veterinarias,
Laboratorio de Inmunoquímica y Biotecnología, Pinto 399, Tandil, Argentina
e-mail: analiain@vet.unicen.edu.ar; paulaluc@vet.unicen.edu.ar; akruger@vet.unicen.edu.ar; nlpadola@vet.unicen.edu.ar

A.B. Bentancor
Universidad de Buenos Aires, Facultad de Ciencias Veterinarias,
Microbiología, Chorroarin 280, CABA, Argentina
e-mail: aben@fvet.uba.ar

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_7

strategies distinguish between those strains that produce animal illness and those that affect humans and have an animal reservoir. The different proposed ways to E. coli control are also discussed.

1 General Concepts

Escherichia coli is a Gram-negative rod that constitutes the intestinal microbiota of animals and humans. However, some strains can cause fatal diseases in humans, mammals, and birds. *E. coli* strains are classified in three distinct groups: commensal strains, intestinal pathogenic *E. coli* strains (InPEC), and extra-intestinal pathogenic *E. coli* (ExPEC) strains, based on genotypic and phenotypic traits (Lyhs et al. 2012).

Genes encoding virulence factors are responsible for the different genome sizes of *E. coli* since commensal and pathogenic strains differ by a million base pairs that correspond to a flexible gene pool (Croxen et al. 2013). These genes are located on genetic mobile elements such as transposons, insertion sequences, bacteriophages, and plasmids, resulting in horizontal gene transfer and within-species genetic variability, which can lead to the differential colonization of hosts (Fig. 7.1). Therefore, it is important to examine health effects across species to understand the animal and public health and to help implement treatment and preventive programs included in the concept One Health (Bidaisee and Macpherson 2014). Further, it is also important to assume the role of the animals in the spread of zoonosis, because if transmission to people is interrupted, the presence of an animal reservoir would remain a continuous risk to people (Ferens and Hovde 2011).

2 Intestinal Pathogenic *E. coli*

Intestinal pathogenic strains are known as diarrheagenic *E. coli* and are grouped into six pathogroups according to virulence factors and diseases they can produce (Hussain 2015): enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) [a subgroup of Shiga toxin-producing *E. coli* (STEC)], enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). There are two other emerging pathogroups: adherent invasive *E. coli* (AIEC) and enteroaggregative enterohemorrhagic *E. coli* (EAHEC) (Clements et al. 2012).

In broad terms, ETEC is the InPEC pathogroup most related to animal disease because it is the causative agent of neonatal diarrhea in farm animals. EPEC and STEC are also often isolated from diarrheic and healthy animals, but their role in

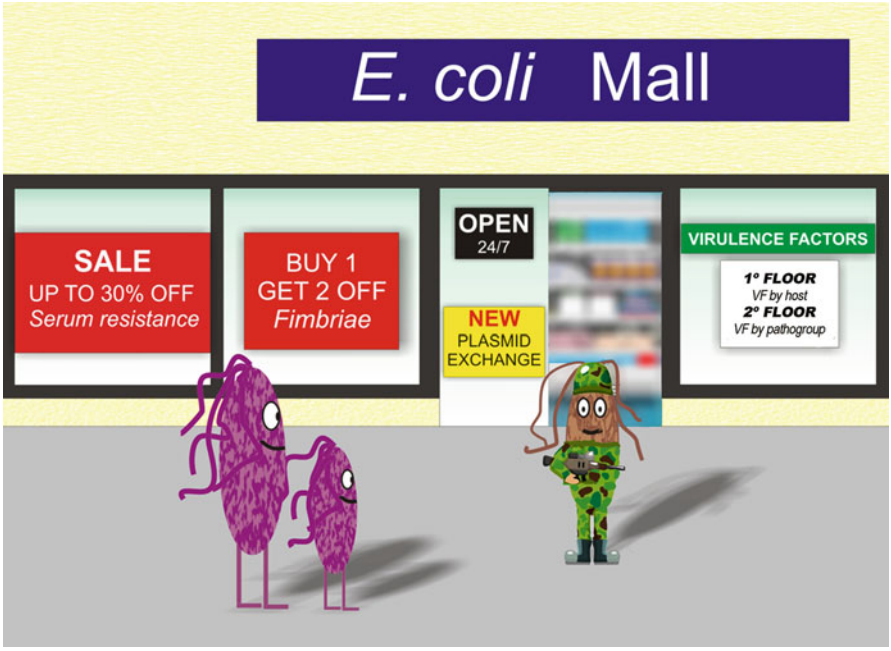

Fig. 7.1 This cartoon aims to highlight the genetic mobility of virulence factors carried by pathogenic *E. coli* strains, which are able to acquire, or lose, factors to adapt to new environments. Although the presence or absence of specific virulence factors is useful for classification of pathogenic *E. coli* strains, it is important to keep in mind the versatility of *E. coli* and their constant evolution

animal disease remains controversial. However, cattle constitute a major carrier of EPEC and reservoir of STEC, which are important human pathogens. EAEC, DAEC, and EIEC are less known in animals (Kolenda et al. 2015).

2.1 Enteroaggregative E. coli

This pathogroup, firstly described in Latin America, can be classified into typical and atypical EAEC based on the presence or absence (respectively) of the *aggR* gene that encodes a protein which regulates several genes linked to EAEC virulence (Okhuysen and DuPont 2010). Animals are not an important reservoir of typical EAEC for human infection, but due to the diversity of EAEC strains, they cannot be definitely excluded (Croxen et al. 2013). Uber et al. (2006) characterized 32 Brazilian EAEC strains from animals with diarrhea (calves, piglets, and horses) and

compared them against human EAEC strains. They identified both typical and atypical EAEC among human strains, but those isolated from animals were all atypical, and they could not find a relationship between human and animal EAEC strains. In addition, in a survey of healthy cattle and pigs in Peru, no EAEC strains were detected using a PCR specific for the *aggR* gene (Rivera et al. 2012). In Brazil (Puño-Sarmiento et al. 2013), EAEC isolates that were *aggR*-positive were identified among *E. coli* isolated from dogs with and without diarrhea, and from one cat without diarrhea, and most isolates showed the characteristic aggregative adherence pattern. Companion animals could either be reservoir for human strains of EAEC or accidental hosts of EAEC since they live in close contact with humans (Hebbelstrup Jensen et al. 2014). Experimental infections in rabbits and rats (ileal loop models) cause lesions similar to those observed in children; therefore, their pathogenicity to these animals should be considered. EAEC has been isolated only rarely from animal sources, and whether animals truly are potential reservoirs of EAEC is not conclusive (Hebbelstrup Jensen et al. 2014).

2.2 *Enteroinvasive E. coli*

There is scarce information about this pathogroup in animals. Few studies have been focused on the detection of EIEC, and furthermore, most of them informed negative results for EIEC markers, although performed in several animal species and countries (Puño-Sarmiento et al. 2013; Chandran and Mazumder 2014). Negative results for EIEC could be due to a low proportion of the analyzed colonies representing this pathogroup, or, on the other hand, to the use of conventional methods to screen *E. coli*, as these strains are frequently late lactose fermenters.

2.3 *Diffusely Adherent E. coli*

This heterogeneous group is a diarrheagenic *E. coli* pathogroup also linked to urinary tract infections in humans (Croxen et al. 2013; Servin 2014). The direct transmission from animals to humans has not been evidenced. Furthermore, methods for DAEC identification are not fully developed because of the occurrence of cross-reactivity of some assays, and the presence of some adhesins both in DAEC and other *E. coli* pathogroups (Servin 2014). There is scarce information about its presence and role in animal disease. In a study performed in different farms in Peru (Rivera et al. 2012), DAEC was not detected among *E. coli* isolated from rectal swabs from healthy cattle and pigs, using a multiplex PCR that detects the *daaD* gene.

2.4 Enteropathogenic E. coli

EPEC strains cause infantile diarrhea by colonizing the intestinal epithelium and then producing effacement of absorptive microvilli from small intestinal enterocytes, leading to attaching and effacement (A/E) lesions. EPEC can also present a mechanism of initial binding to host epithelial cells mediated by a type IV pilus encoded in the EPEC adherence plasmid. Depending on the presence or absence of these pili, these strains are classified as typical and atypical, respectively (Hussain 2015). Atypical EPEC (aEPEC) have been considered an emerging pathogen in developed countries, producing endemic diarrhea in children as well as outbreaks, although recent data suggests that aEPEC are more prevalent than typical EPEC (tEPEC) in both developing and developed countries (Santona et al. 2013).

The aEPEC strains have been found in a variety of animal reservoirs including bovines, rabbits, monkeys, dogs, birds, and cats, while the main reservoir for tEPEC are humans (Croxen et al. 2013).

EPEC may cause intestinal diarrheal disorders in animals. However, healthy chickens may carry EPEC strains, suggesting that chicken could be a reservoir of these bacteria. In Argentina, aEPEC was isolated from 12 % of live animals (cloacae), and with variable prevalence from eviscerated carcasses, washed carcasses, and water from chillers at different stages of the slaughtering process. The isolates presented a wide variety of serotypes, some of which had been reported in other animal species (O2:H40, O8:H19, and O108:H9), while O45:H8 had been previously isolated from children (Alonso et al. 2016).

In wild birds from Canada, Chandran and Mazumder (2014) have found 15 % of prevalence of EPEC, while in urban feral pigeons from Peru, EPEC represented 5.8 % of *E. coli* isolates (Caballero et al. 2015) and was detected in 12 % of feral pigeon droppings from Brazil (Silva et al. 2009). tEPEC were isolated from 11 of 18 diarrheic alpacas in Peru, while the remaining were only *eae* positive (aEPEC), suggesting that alpacas harbor potentially pathogenic strains that might cause clinical and fatal intestinal disorders in young animals (Luna et al. 2012).

Jay-Russell et al. (2014), in Mexico, isolated aEPEC strains from 3.6 % of dog and 4.9 % from coyote samples belonging to 14 different serotypes. In Brazil, the *E. coli* pathogroup most prevalent in dogs and cats was EPEC. The ONT:H16 serotype was predominantly found in isolates from dogs with diarrhea, although EPEC O88:H25, ONT:H5, ONT:H6, and ONT:H12 were also isolated from dogs. In Argentina, Bentancor et al. (2010) isolated aEPEC O157:H16 and tEPEC O157:H45 from household dog samples. Interestingly, the aEPEC O157:H16 serotype appears to be part of a large clonal group that is prevalent worldwide (Feng et al. 2012).

Considering calves as reservoirs for human pathogenic *E. coli*, EPEC were found more often in healthy than in diarrheic animals with 14.6 % of strains isolated from healthy and 7.5 % isolated from diarrheic animals (Kolenda et al. 2015). In Peru, EPEC were detected more frequently in cattle (18 %) than in pigs (5 %) and mainly from cattle younger than 24 months of age than in older cows (21 % vs. 13 %) (Rivera et al. 2012).

In calves, EPEC appears not to be the main diarrheagenic pathogen, since it is shed during a short period of time, resulting in a low prevalence. Epidemiological studies in cattle from Brazil have demonstrated that 1.4–42.5 % of diarrheic cattle eliminated EPEC (Coura et al. 2014). However, EPEC plays an important role as causal agent of colibacillosis, and its incidence and impact on morbidity/mortality of newborn calves might be relatively high (Bartels et al. 2010).

In sheep from Brazil, EPEC isolates that carry genes associated with diarrhea in humans were detected. Thus, Maluta et al. (2014) using markers recently associated with disease demonstrated that EPEC strains similar to those pathogenic for humans are present in the sheep intestinal microbiota, underlining the potential for food-borne transmission.

Croxen et al. (2013) suggested interspecies transmission as a means for human infection with aEPEC and rabbits as a possible animal reservoir. In rabbits, natural EPEC infection causes profuse watery diarrhea that can be mucoid or bloody, accompanied by anorexia, dehydration, and lethargy.

Very little is known about the occurrence of EPEC in synanthropic rodents. In Argentina, Blanco Crivelli et al. (2013) characterized aEPEC from *Rattus rattus* and *Rattus novergicus* captured in the urban area of Buenos Aires city and suggested that synanthropic animals could be natural reservoir of EPEC.

2.5 Enterotoxigenic E. coli

ETEC strains are an important cause of diarrhea in farm animals, such as calves and piglets, but they are very rare or absent in horses, rabbits, and poultry. The main virulence factors of ETEC strains are enterotoxins and adhesins, but other less characterized factors have also been described in some strains of this patho-group. During infection, ETEC first adhere to small intestinal epithelium and then secrete the enterotoxins that disturb enterocyte function. Host specificity (e.g. species and age) of ETEC can be mostly explained by differences in expression levels or presence of adhesin receptors (Gyles and Fairbrother 2010). The adhesins most frequently found in ETEC from pigs are F4 (K88), F5 (K99), F6 (987P), F18, and F41 fimbriae, and afimbrial adhesins such as AIDA-I. Among ETEC from calves and lambs, the most important adhesins are F5 (K99) and F41. Differential expression of some adhesin receptors on the intestinal epithelium between adult and newborn animals can explain why certain types of adhesins are mostly associated to diarrhea in young animals than in adults. However, there are also uncharacterized adhesins which may play a role in ETEC pathogenesis, since some ETEC strains which produce enterotoxins but lack recognized fimbria have been described (Gyles and Fairbrother 2010).

Enterotoxins secreted by ETEC comprise two major classes: heat-stable (ST) and heat-labile toxins (LT). ST comprises the STa (or STI), STb (or STII), and EAST1 (related to STa) subtypes, and LT includes the LT-I and LT-II subtypes (Gyles and Fairbrother 2010). Like ETEC adhesins, most enterotoxins are encoded by plasmids.

In pigs, studies performed in Argentina identified different combinations of ETEC virulence genes among *E. coli* from non-diarrheic pigs. One of the studies, with 7–15 day-old piglets, reported a higher proportion of *E. coli* isolates carrying only one detected ETEC virulence gene, such as the toxins STb or LT, or the adhesins P987, F4, or F18 in comparison to isolates carrying a toxin gene along with an adhesin encoding gene (STb/F18 profile) (Alustiza et al. 2012). A study with pigs from different production stages assessed the presence of ETEC by PCR targeting the genes e*ltA* (LTa) and *estI* (STa) and showed that 15.2 % of the animals were carriers of *E. coli* with either one or both toxin genes (Moredo et al. 2015). The highest percentage of animals positive for ETEC was observed in the farrowing phase (66 %). Although not searched in the screening, the subsequent characterization of the isolates showed that a high proportion of them were *estII* positive (encoding STb) (97.5 %) and 16 different virulence profiles were detected among the 40 ETEC isolates. Among them, genes encoding adhesins F5, F6, F18, and AIDA-I were identified. Some of the isolates carried the gene encoding the EAST1 toxin and others the *stx*2e gene, thus showing the presence of ETEC/STEC hybrid strains. In Brazil, Cruz Junior et al. (2013) found no difference between diarrheic ($n = 30$) and non-diarrheic newborn piglets ($n = 30$) regarding the frequency of ETEC carriage. They detected genes coding for F41, 987P (F6), and LT, but only two isolates harbored an adhesin plus the enterotoxin gene.

Andrade et al. (2012) characterized *E. coli* isolated from diarrheic and non-diarrheic calves with 6–60 days of age and found ETEC only in animals without diarrhea. They also identified isolates that carried only the F41 encoding gene in both groups of animals. A longitudinal study of calves in Brazil found a low frequency of ETEC with no association with diarrhea (Coura et al. 2015). These authors reported the presence of ETEC [virulence profile STa F5 (K99)] in diarrheic calves up to 16 days of age (with the exception of one calf). No significant differences in ETEC prevalence between healthy and diarrheic calves (of less than 10 days of age) were reported by Picco et al. (2015) in Argentina. In that study, the genes for the adhesins F5, F17, and F41 were detected, F17 being the most prevalent gene (alone or in combination with other genes). The only enterotoxin gene observed was the one encoding STa (alone or in combination with adhesin-encoding genes), and several isolates were positive for the presence of adhesins but negative for enterotoxins.

In a study performed on *E. coli* isolated from fecal samples of lambs (7–10 days of age) in the same country, 48 % of the animals carried *estA*-positive strains of which all were negative for F41 and K99 genes and only two carried the F17 gene (Aragão et al. 2012).

2.6 Shiga Toxin-Producing E. coli

STEC are zoonotic pathogens that cause the vascular endothelial damage observed in patients with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) by production of Shiga toxins (Stx1 and Stx2), encoded by *stx1* and *stx2* genes, respectively. Another typical virulence factor is intimin (*eae* gene), which is required for intimate bacterial adhesion to epithelial cells inducing a characteristic histopathological A/E lesion, governed by locus of enterocyte effacement (LEE). Strains lacking *eae* are named as LEE-negative STEC and have been also associated with severe disease in human. In this group, the overall genome content, phage location, and combination of potential virulence factors are variable. An autoagglutinating adhesin (Saa), encoded by a plasmid, could be involved in the adhesion of these strains that do not carry *eae* (Padola and Etcheverría 2014). Additional plasmid-encoded virulence genes associated to STEC are an enterohemolysin (*ehxA*), a catalase-peroxidase (*katP*), an extra-cellular serine protease (*espP*), a zinc metalloprotease (*stcE*), a subtilase cytotoxin (*subAB*), among other proteins with adherence and colonization functions.

Regarding serotypes, and due to the importance of serotype O157:H7 in human disease, it is common to divide STEC serogroups in two major categories, O157 and non-O157, cattle and other animals being the reservoirs for both categories.

Cattle are the natural reservoir of STEC, but other ruminant species such as sheep, goats, and deer may also act as reservoirs, shedding these bacteria through their feces, spreading and maintaining these pathogens among cattle herds and the environment. Animals could maintain STEC carriage in the absence of continuous exposure or require frequent re-exposure to STEC from environmental sources with potential interspecies and intraspecies infection (Etcheverria and Padola 2013; Persad and LeJeune 2014).

In Argentina, the country with the highest worldwide incidence of HUS, 545 STEC isolates obtained from 4824 samples from cattle, foods (hamburger and minced meat), and environment of farms were characterized. There were serotypes shared between cattle and foods (O8:H19, O91:H21, O113:H21, O117:H7, O130:H11, O157:H7, O171:H2, and O178:H19) and also between cattle and the environment (O8:H19, O26:H11, and O145:NM). Among calves, the profiles *stx1*/*eae*/*ehxA* and *stx2*/*eae*/*ehxA*, and *stx2* were the most frequent, and these three profiles also predominated among environmental STEC strains (Etcheverria and Padola 2013). In addition, a considerable proportion of the STEC isolates from cattle in Argentina showed to carry *stx*-subtypes associated with severe disease in humans (Krüger et al. 2011).

Several studies informed variable prevalence of STEC ranging from 22 to 67 % in cattle from Argentina. Fernández et al. (2012) found STEC-positive animals in 25 %, 43 %, and 58 % of newborn, milk-fed, and growing dairy calves, respectively. The presence of STEC in newborn calves less than 24 h old suggested that they are exposed to this bacterium quickly after birth and play an important role in vertical STEC transmission. Low prevalence of STEC O157 was detected in milking cows (0.2 %) and calves (0.8 %).

STEC O130:H11 and O178:H19 were the most prevalent serotypes isolated from dairy cows (Fernández et al. 2010), from beef abattoirs (Masana et al. 2011), and from feedlot cattle (López et al. 2012) from Argentina. Both serotypes have been isolated from HC and HUS cases in several countries. With regard to STEC strains belonging to the O26:H11 serotype, they were detected in different animal categories, and noticeably, an important proportion of these strains carried the *stx2a* subtype (with no other *stx*-subtype), differing from those circulating in Europe or USA (Krüger et al. 2015).

In Brazil, Freitas Filho et al. (2014) detected a prevalence of 1.9 % of O157:H7 among dairy calves, while in USA, STEC O157 was found on almost all cattle farms, with the organism being shed intermittently by most animals (Persad and LeJeune 2014).

Regarding the association of STEC carriage and diarrhea in cattle, the studies are controversial. While Kolenda et al. (2015) performed a systematic review and meta-analysis finding no association between STEC and diarrhea, Coura et al. (2015) found statistical association between diarrheic animals carrying *E. coli stx1*/*eae* in their feces at 2 and 4 weeks of age and *E. coli stx2* at 5 weeks of age, suggesting that STEC could cause diarrhea in calves older than 2 weeks.

Transmission of STEC to small ruminants occurs through the same pathway as in cattle, but the site of STEC colonization may be different since tropism for the recto-anal junction (RAJ), as occurs in cattle, has not been described for all small ruminants (Persad and LeJeune 2014).

STEC (O157 and non-O157) have been isolated from other captive and wild non-domesticated ruminant species, including llamas, moose, alpacas, antelopes, and yaks. These animals can transmit STEC to humans directly by contact at petting zoos or indirectly through fecal contamination in water sources, vegetable fields, recreational areas, or on meat (Persad and LeJeune 2014).

Species of deer, including red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*), have also been identified as capable of shedding STEC. Persad and LeJeune (2014) reviewed that in elk (*Cervus canadensis*) STEC serogroups O103 and O146 were detected and the prevalence in elk feces was found to be higher than in mule deer.

Several studies have been conducted to detect STEC in Alpacas from Peru. Luna et al. (2012) detected STEC in 45.5 % of alpaca samples, while Silvera et al. (2012) isolated seven strains *eae* and four *stx2* in alpacas without diarrhea, and two strains *eae* and *stx1*, one *stx2*/*eae* and, one *stx1* and five *stx2* in alpacas with diarrhea. STEC O157 was not detected in these studies.

Swine also play an important role as carrier of STEC strains. Some STEC strains can produce Stx2e, the subtype related to porcine edema disease. The toxin binds epithelial and endothelial cells, impairing blood vessels, leading to edema, ataxia, and death. Risk factors, including dietary changes and the introduction of pigs to new herds, have been suggested for edema disease onset. In Argentina, Colello et al. (2016) conducted a study through the pork chain production, suggesting a vertical transmission of STEC. At farms, 2.8 % of samples were *stx* positive; at slaughter, 4.08 % of carcasses were *stx* positive; and at boning rooms, 6 % of samples were *stx*

positive, detecting that isolates carried *stx1*/*stx2* genes, *stx2e,* and some of them only *stx2*. Regarding *stx2e*, its prevalence decreased from pigs at farms to pork meat. In Brazil, from 800 samples collected, *E. coli* were identified in 561 samples, with 16.0 % carriers of *stx1*, 17.3 % of *stx2,* and 8.0 % of *eae* (Assumpção et al. 2015). Several studies have been conducted to isolate O157:H7, reporting prevalence up to 10 %. In the United States, this prevalence usually is less than 2 %. A study in Canada described an outbreak of STEC O157:H7 infection linked to consumption of pork, highlighting the risk associated (Trotz-Williams et al. 2012).

The published data on the prevalence of STEC in horses and donkeys indicate that they are no major reservoirs of STEC and may instead be spillover hosts (Roug et al. 2013). Regarding birds, the STEC prevalence levels are low, although their potential to transmit STEC to other birds and contaminate the environment is a serious risk (Persad and LeJeune 2014). In recent years, the population growth of feral pigeons has increased public health concern, because they could represent a reservoir of transmissible pathogens. In Peru, 0.98 % of feral pigeons were carrier of STEC (Caballero et al. 2015), while in Brazil a STEC frequency of 4.6 % was found in birds.

Since the middle of the twentieth century, pets are more frequently considered as "family members" within households. However, cats and dogs still can be a source of human infection due to various zoonotic pathogens (Chomel 2014). Household pets have been confirmed as carriers of STEC in metropolitan areas. Bentancor et al. (2007) evaluated household dogs and cats from Buenos Aires city with and without diarrhea and all STEC isolates recovered from dogs harbored the *stx2* sequence. The major serotype was O178:H19, followed by O91:H16, O91:H21, O157:NM, and O8:H19. Two STEC isolates belonging to the serotypes O91:H16 and O8:H19 were recovered from one dog fecal sample. Puppies up to 2 years of age presented significantly higher infection prevalence than older pets. None of the strains possessed the *subAB*, *eae,* or *saa* genes and only one strain of serotype O8:H19 carried the *ehxA* gene. Besides, an O91:H21 strain from dog was confirmed to carry the subtype *stx2d*, formerly mucus-activatable toxin. Furthermore, all those dog STEC strains showed cytotoxic activity on Vero cells. Cats evaluated by Bentancor et al. (2007) showed a higher prevalence of STEC than dogs, and all of the isolates were *eae*-positive belonging to the serotypes O22:H8 and O8:H19. Besides, O8:H19 strains carried the *ehxA* gene and expressed the enterohemolytic phenotype. O8:H19 strains had been previously isolated from three clinical cases of HUS in that country, but did not match the strains isolated from pets. Noticeably, circulating O22:H8 strains from cat, bovine, and hamburger showed 100 % similarity among them (Bentancor et al. 2011).

It is important to note that the proportion of STEC carriers increased when the evaluated household pets were related to HUS cases. Moreover, one O145:NM strain was isolated from a healthy household cat, which was related to a HUS case (Rumi et al. 2012). STEC carriage in pets related to a HUS case was detected up to 15 days from their first isolation (Bentancor 2016).

Most of the STEC serotypes isolated from pets in Argentina were previously recovered from cattle and/or associated with cases of severe illness in humans. A great proportion of the strains identified were closely related to strains of the same serotype isolated from cattle, meat, or humans in the same or in distant geographical

areas. Among 61 O178:H19 STEC strains from Argentina and Germany, three Argentine strains from dogs showed high similarity (94.4–100 %) to strains from cattle and food of bovine origin, although no epidemiological relatedness according to space-time pattern was identified (Miko et al. 2014).

Blanco Crivelli et al. (2012) showed the first worldwide finding of STEC in *R. rattus* and reported STEC non-O157 circulating in different synanthropic rodent species from Argentina. None of the strains belonged to the O157 serogroup, which is the most frequently associated with HUS. However, the STEC O174:H21 isolated from *R. rattus* belongs to one of the four prevalent non-O157 serogroups in Argentina.

Rabbits have also been used as a possible animal model to study STEC infection in humans, since they demonstrate enteric and renal lesions when challenged with STEC (Persad and LeJeune 2014).

3 Extra-Intestinal Pathogenic *E. coli*

ExPEC cause diseases in animals, although it is difficult to confirm whether strains from animals have the potential to cause human disease. These strains can produce urinary tract infections, newborn meningitis, sepsis, septicemia, airsacculitis in poultry, mammary and endometrial infections and they have been isolated from poultry, cattle, swine, dogs, cats, horses, and wild animals (including rats and birds) (Bélanger et al. 2011; Kunert Filho et al. 2015).

According to the diversity of clinical signs and the different virulence factors, ExPEC is divided in pathogroups: uropathogenic *E. coli* (UPEC), sepsis/newborn meningitis associated *E. coli* (NMEC), avian pathogenic *E. coli* (APEC), sepsis-associated pathogenic *E. coli* (SePEC), mammary pathogenic *E. coli* (MPEC), endometrial pathogenic *E. coli* (EnPEC) (Kunert Filho et al. 2015), and necrotoxigenic *E. coli* (NTEC) (Croxen and Finlay 2010).

In animals, ExPEC cause diseases, and also it is important to take into account the antimicrobial resistance of these strains, because it can increase the incidence of animal and human infections and complicates their treatment (Bélanger et al. 2011).

3.1 Mammary Pathogenic E. coli

E. coli are one of the causes of mastitis worldwide and no association with human disease has been reported for strains that cause bovine mastitis (Bélanger et al. 2011). A mammary pathogenic *E. coli* (MPEC) pathogroup has been suggested by Shpigel et al. (2008) for strains that cause mastitis in dairy animals, but they can also be included in the more general ExPEC pathogroup. Recent studies by Kempf et al. (2016) intended to identify genomic characteristics that could support the hypothesis of MPEC as a distinct pathogroup. They observed a poor content in virulence genes of mastitis strains, which became clustered

with a non-pathogenic *E. coli* strain and differed from strains belonging to InPEC and ExPEC pathogroups. However, they could not find genotypic traits specific for mastitis isolates. In accordance to Kempf et al. (2016), Bentancor and Gentilini found few virulence-associated genes in 25 MPEC isolates from Argentina (unpublished data).

The existence of differences in disease presentation and severity, the diversity of MPEC, and the fact that dairy cows are highly inbred point to the hypothesis that MPEC carry different combinations of virulence genes that could explain differences in disease (Shpigel et al. 2008). In a study of different Brazilian mastitis isolates, the *fimH* gene was detected in all the isolates, in different combinations with other virulence genes and it was concluded that more than one factor participates in the pathogenicity of these strains (Fernandes et al. 2011).

There are also some studies aimed to identify characteristics that could differentiate MPEC isolates associated to transient mastitis from those of persistent mastitis. Dogan et al. (2012) found that both transient and persistent isolates (from USA and The Netherlands) had a high genetic heterogeneity, and they could not identify genes that could characterize each group of isolates. Interestingly, these authors found an association between the presence of *lpfA* and increased epithelial invasion of bovine mammary epithelial cells. In addition, *lpf* gene has been also identified in some *E. coli* isolates from bovine mastitis in Chile (Cartes Lillo 2014).

3.2 Avian Pathogenic E. coli

This section highlights some characteristics of APEC; for further details, see Chap. 9. APEC comprise those *E. coli* strains that cause extra-intestinal infections (colibacillosis) in avian species (Mellata 2013) and are considered a major pathogen within the poultry industry.

APEC is a heterogeneous group, and the mechanisms of virulence that define APEC are not completely elucidated. APEC have multiple virulence genes involved in different steps of infection and/or fitness, such as those encoding for factors associated to adhesion, iron acquisition, serum resistance, virulence regulation, and other virulence traits (Kunert Filho et al. 2015).

The prevalence of different virulence-associated genes has been evaluated in several studies. Plasmid-carried genes (such as *cvi*/*cva*, *iroN*, *iss*, *iucD,* and *tsh*) have been frequently detected, but with variable prevalence in APEC strains collected in Brazil (Cunha et al. 2014). Interestingly, Prioste et al. (2013) isolated *E. coli* strains from 46 of 87 cloacal fecal samples obtained from asymptomatic *Guaruba guarouba*, and 61 % of the isolates harbored at least one of the genes commonly reported in poultry (*irp*2, *iucD*, *iss*, *vat*, *cvi*/*cva*, *tsh*, and *astA*). Additionally, Saviolli et al. (2016) recovered some *E. coli* strains from samples of free-ranging frigates, apparently healthy, in Brazil, that had a combination of five genes regarded as minimal predictors of virulence for APEC (*iroN*, *hlyF*, *ompT*, *iss,* and *iutA*).

Although a proper diagnosis of avian colibacillosis requires isolation and identification of *E. coli* from birds with clinical suspicion of disease, the diversity among APEC strains hinders a clear identification of an avian *E. coli* isolate as a pathogenic. In recent years, big efforts were made to define APEC and determine molecular markers for its identification by genotyping methods. However, and taking into account that an isolate cannot be clearly defined as APEC based solely on genotypic profile, confirmation of virulence in animal models is suggested (Mellata 2013).

3.3 Uropathogenic E. coli

This section highlights some characteristics of UPEC; for further details, see Chap. 9.

E. coli is the most common uropathogen in dogs and cats (Chew et al. 2011). Urinary tract infections (UTI) are common in dogs, but bacterial UTIs in cats are relatively uncommon (Chew et al. 2011). In Chile, a study of 48 clinical records of dogs and cats with UTI showed that *E. coli* was the most frequent bacterium isolated from urine samples, and similar results were reported in Brazil by Carvalho et al. (2014). In The United States, Wong et al. (2015) identified *E. coli* in 58.1 %, 57.7 %, and 49.3 % of 1636 aerobic bacterial isolates recovered from urine samples from dogs with uncomplicated UTI, complicated UTI, and pyelonephritis, respectively.

Although previous studies had reported that *sfa*, *cnf*, *hly*, and *pap* genes are frequently found in canine UPEC, Wells et al. (2013) showed that 89 out of 159 (52 %) *E. coli* strains isolated from canine patients in USA did not possess any of these genes. In Argentina, Cundon et al. (In Press) characterized UPEC in 69 dogs and in 15 cats. They detected combinations of *pap1/2*, *pap3/4*, *sfa,* and *cnf* only in 12 % out of 84 strains and did not found *hly*.

Osugui et al. (2014) identified *E. coli* as the etiologic agent of UTI in 7/14 (50 %) and 36/86 (42 %) of urine samples from cats and dogs in Brazil. This study showed that *fimH* was present in all isolates, while the *iha*, *sfa*, *cnf1,* and *hlyA* genes were present only in strains belonging to phylogenetic group B2. In Argentina, Cundon et al. (In Press) also found *sfa* and *cnf1* in UTI strains belonging to phylogenetic groups A0 and ExPEC B2 and D from felines and canines, and in accordance with Osugui et al. (2014), was absent in B1.

Recently, Liu et al. (2015) characterized 74 UPEC strains isolated from cats in four geographic regions of USA. The study showed B2 as the predominant phylogenetic group and extremely diverse profiles of virulence-associated genes. However, among strains belonging to phylogenic group B2, some genes, such as *hlyD*, *hly*A, *cnf1,* and *iroN,* were frequently detected.

Swine can be also affected by UPEC strains. Urinary infections affect mainly adult animals (Kunert Filho et al. 2015).

3.4 Endometrial Pathogenic E. coli

Most cattle are affected by ascending infections after parturition. In some cows, bacteria persist in the uterine lumen and can favor the development of postpartum uterine diseases such as metritis and endometritis (Kassé et al. 2016).

E. coli is one of the pathogens commonly isolated from samples of cattle with metritis and endometritis; however, its role in the pathogenesis is still poorly understood (Bicalho et al. 2012; Wagener et al. 2014). In a study of 374 lactating cows from farms in Nueva York, Bicalho et al. (2010) found that 33.4 % of the samples were positive for *E. coli*. Molecular characterization of *E. coli* isolates showed that six virulence-associated genes (*fimH*, *astA*, *cdt*, *kpsMII*, *ibeA*, and *hlyA*) were significantly associated with the incidence of metritis and endometritis. In particular, *fimH* was highly prevalent in *E. coli*-infected cows and was an important predictor of uterine disease. In a later study, Bicalho et al. (2012) evaluated the virulence factors present in the uterus of cows at three different stages of lactation and observed a strong association of *fimH* with metritis and clinical endometritis in the first days, but not at later stages of lactation, suggesting that *E. coli* is likely among the first bacteria to colonize the intrauterine environment, potentially inducing changes that will favor colonization by other pathogens.

A study from Canada evaluated the prevalence of *E. coli* in the uterus of postpartum dairy cows before the onset of postpartum metritis and showed that cows positive for intrauterine *E. coli* were three times more likely to have subsequent postpartum metritis compared with bacteriologically negative cows. In addition, the *hra1* and *kpsMTII* genes were also associated with greater odds of postpartum metritis (Kassé et al. 2016).

3.5 Sepsis-Associated Pathogenic E. coli and Neonatal Meningitis E. coli

In some circumstances, *E. coli* strains can traverse the intestinal epithelial barrier and adapt to extra-intestinal conditions. It is considered that SePEC strains must have several factors that enable them to invade the host by an entry point, resist bactericidal host effects, persist and multiply in the blood, and other extra-intestinal sites (Fecteau et al. 2009). However, they are not characterized by a particular virulence factor or group of factors. SePEC virulence factors detected in calves and pigs are variable among strains and include F17, P and S fimbrial adhesins, colicin V, CNF1 or CNF2, CDT (Gyles and Fairbrother 2010).

E. coli septicemia occurs more frequently in newborn animals. It is the cause of neonatology morbidity and mortality, and it is responsible for significant economic losses in animal production (Kunert Filho et al. 2015). *E. coli* have been reported as the predominant organism isolated from septicemic calves and neonatal pigs (Gyles and Fairbrother 2010).

Neonatal bacterial meningitis and bacterial sepsis are often linked. *E. coli* is also reported to be the most common bacterial isolate in calves and foals with meningitis. The income of the bacterium into the meninges is poorly understood. Some *E. coli* virulence factors may be important to resist defense mechanisms, reach the cerebrospinal fluid, and replicate (Fecteau et al. 2009).

3.6 Necrotoxigenic E. coli

Necrotoxigenic *E. coli* (NTEC) are responsible for various diseases of humans and animals, including urinary tract infection, septicemia, and diarrhea. NTEC possess cytotoxic necrotizing factors (CNF1, CNF2 or CNF3) (Orden et al. 2007), as well as a cytolethal distending toxin (CDT). Some or all of these pathovars can also express other virulence factors such as intimin (Croxen and Finlay 2010). The subgroup of NTEC known as NTEC-2 has broad distribution among production animals and produces the variant of CNF (CNF-2) whose gene is located on a plasmid known as Vir.

4 Control

For control of *E. coli* infections, it is necessary to distinguish between those strains that produce animal illness and those that affect humans via the food chain being animals their reservoirs. Vaccines and antibiotics have been essential in the control of infectious disease for many years. However, for several diseases, there are no effective vaccines or antibiotics available.

4.1 InPEC Control

Control of STEC may prevent human illness by reducing the presence of these pathogens throughout the beef production chain. There are several strategies to reduce the shedding of STEC in cattle feces, such as vaccination, probiotics (as Direct Fed Microorganisms-DFM), and bacteriophages, among others. While it is broadly shown that cattle are carriers of STEC O157 and non-O157, most of studies attempting to control cattle as reservoir have been directed towards O157. Thus, the effects of possible interventions on STEC non-O157 must be deduced from the available information on STEC O157:H7 control (Gill and Gill 2010). While post-harvest pathogen-reduction strategies have been largely successful at reducing direct food-borne illness, these processing interventions have not been perfect.

Research into pre-harvest pathogen reduction controls and interventions has grown in the last years. Strategies that specifically target food-borne pathogenic bacteria, as STEC, in the animal at the farm level have great potential to improve food safety and decrease human illnesses (Sargeant et al. 2007).

Vaccination of cattle, the major reservoir for STEC, could be an effective public health control against a serious disease, although studies that do not account for nonlinearity in cross-species transmission may substantially underestimate the efficacy of interventions against zoonotic pathogens (Hurd and Malladi 2012).

There is a systematic review that concluded that vaccination of cattle has efficacy as a pre-harvest intervention (Snedeker et al. 2012). As reviewed by Callaway et al. (2013), some vaccines had been developed, one based on type III secreted proteins (TTSP), other using EspA, intimin, and Tir, involved in STEC adherence. Several studies have demonstrated that vaccinating cattle using TTSP decreases the probability of detecting STEC O157 from cattle feces (Wisener et al. 2015). Other study suggested that TTSP and SRP (siderophore receptor and porin protein) vaccines significantly reduce fecal prevalence of *E. coli* O157 in cattle. However, especially for the SRP vaccines, there have still been relatively few trials published. Future research would be of interest, particularly in feedlot or research settings where cattle are at production density (Snedeker et al. 2012). Systemic vaccination with *E. coli* O157 BGs (bacterial ghosts) provides protection in a bovine experimental model (Vilte et al. 2012). Rabinovitz et al. (2012) found specific antibodies in colostrum which were transferred efficiently to newborn calves by feeding colostrum from cows immunized with EspB, the C-terminal 280 amino acids of γ-Intimin (γ-Intimin C280), and inactivated Stx2 proteins fragment. This can be an alternative to protect calves' early colonization by STEC O157:H7 and a possible key source of antibodies to block the colonization and toxic activity of this bacterium.

Commercial vaccines have been available and have been proved to significantly reduce O157 in cattle digestive systems by 50–75 % on average, with some cattle showing reductions as high as 98 %. Currently, there are two vaccines commercially available directed against *E. coli* O157—Canada's Econiche® and America's Epitopix SRP®. Both are under limited licensing in the USA, but Econiche® has been fully licensed in Canada (Zuraw 2013).

Probiotics as DFM have been used as potential pre-harvest interventions for the reduction of food-borne bacterial pathogens such as O157. As for the competitive strategies, the use of probiotic bacteria as competitive microbiota has shown promising results in controlling STEC. Probiotics can interfere with pathogenic strains by producing metabolites that are inhibitory to STEC O157:H7 and non-O157. Some strains of *E. coli* can produce colicins that are inhibitory in vitro to DEC strains. Several authors have identified bacteria with potential ability to exclude STEC O157:H7 from the gastrointestinal tract of cattle (Etcheverria et al. 2006; Sargeant et al. 2007).

Many studies have reported that supplementing with *Streptococcus faecium* the diet of animals that are infected with *E. coli* O157:H7 reduces its shedding in feces. However, more effective reduction in *E. coli* O157:H7 shedding can be obtained by treatment with multiple probiotic bacteria consisting of *Enterococcus faecium*,

Lactobacillus acidophilus, *L. casei*, *L. fermentum,* and *L. plantarum*. Reduction in *E. coli* O157:H7 shedding by ruminants decreases the contamination chances of meat and other food products, thereby decreasing the potential for *E. coli* O157:H7 outbreaks. Probiotic cultures based on diverse bacterial strains (*Lactobacillus gallinarum*, *Streptococcus bovis*, *S. faecium* or a mixture of *S. faecium*, *L. acidophilus*, *L. casei*, *L. fermentum* and *L. plantarum*, *Propionibacterium freudenreichii*, among others) reduced *E. coli* O157:H7 shedding in cattle (Callaway et al. 2013). Studies have also indicated that cultures of *Lactobacillus acidilacti* and *Pediococus* could directly inhibit *E. coli* O157:H7, likely through the production of organic acids and low pH (Rodriguez-Palacios et al. 2009).

Regarding phages as an alternative strategy for control, it has been examined for use in two different approaches to reduce *E. coli* O157:H7, within the gut of cattle before slaughter, and as a hide or environmental decontaminant (Ricke et al. 2012). Phage products for use as a hide spray have been released into the marketplaces (Finalyse®). Bacteriophages have been used to control experimentally inoculated food-borne pathogenic bacteria in cattle gastrointestinal tracts (Callaway et al. 2013). Other authors have concluded that a continuous phage therapy can be an effective method to reduce *E. coli* O157: H7 carriage (Rozema et al. 2009).

The most important factor, in the case of newborn animals, to take into account when preventing ETEC infections, is an early and sufficient colostral supply. The protective value of colostrum against diarrheal diseases of the newborn caused by ETEC can be increased essentially by maternal immunization. Several vaccines are used mainly for parenteral application containing protective antigens (virulence factors—fimbrial adhesins with or without LT enterotoxins). Colostral antibodies would block virulence factors and propagation of bacteria in the intestine. Similar effects can be expected in the case of passive immunization, e.g., the oral application of polyclonal or monoclonal antibodies (Zhang 2014).

To prevent diarrheal disease due to ETEC in pigs, there are several important tools as management techniques and good farming practice. There are some factors that must be taken into account as strategies to prevent diarrheal disease. These factors include weaning age and weight, weaning diet, overstocking, and contaminated environment from earlier stocks. Vaccinations against neonatal diarrhea due to ETEC have been very successful, especially since the most prevalent adhesins (K88, K99, 987P) and toxin (LT) became standard components of the vaccines. Some researchers demonstrated the efficacy of live oral vaccines applied before weaning. Commercial vaccines for sows contain killed *E. coli* F4, F5, F6, and/or F41 fimbriae, either purified or as inactivated *E. coli* expressing these fimbriae with or without the LT toxoid (Cox et al. 2014). There is a commercial vaccine introduced in Brazil in 2011 containing naturally avirulent *E. coli* bacteria that express F4 fimbriae, but do not produce toxins. Clinical studies have demonstrated significantly reduced colonization of pigs' intestines after challenge (Cox et al. 2014).

Some approaches, including treatment with antibiotics, passive administration with specific antibodies, dietary supplementation including prebiotics, and probiotics, along with vaccine development, to control post-weaning diarrhea have been attempted. These prevention approaches, as an alternative for vaccination, have

shown some promise, but cost-effectiveness or concerns of environmental risk make them less favorable or not practical (Zhang 2014). Regarding the use of probiotics in ETEC control, some studies have found that the use of a mixture of the *L. casei* culture with maltodextrins promoted a significant reduction of a pathogenic *E. coli* into the jejunum of gnotobiotic pigs. Other study has reported that the use of spores of *Bacillus licheniformis* together with *B. cereus* var. toyoi has resulted at effective reducing of diarrhea, mortality, and weight loss in weaned pigs. The selection of probiotic *E. coli* strains against *E. coli* K88 is effective in preventing diarrhea in piglets when fed in conjunction with raw potato starch and in reducing the negative effects of ETEC in a piglet challenge model (Krause et al. 2010).

For the immunization to mastitis, a vaccine containing an *E. coli* O111:B4 (named J5) and *S.* Typhimurium Re-17 was employed. The immunization with this vaccine was capable of diminishing the number of clinical cases and improved milk production (Gentilini et al. 2013).

4.2 ExPEC Control

Because of the extensive use of antibiotics, there is an increased population of strains that developed antibiotic resistance. Reducing the animal reservoir as source of ExPEC using vaccines to replace or reduce antimicrobials could diminish losses due to ExPEC and limit the spread of these pathogens to humans (Bélanger et al. 2011).

The prevention and control of APEC infections include the control of environmental contamination and environmental parameters such as humidity and ventilation. APEC are frequently resistant to a wide range of antibiotics; nevertheless, antibiotic therapy is widely used. Vaccines containing killed, attenuated virulent bacteria showed protection against infection with the homologous strain, but are less efficient against heterologous strains. There are some subunits, and recombinant vaccines used to immunize chickens against colibacillosis (Kunert Filho et al. 2015). There is a commercial vaccine (Poulvac®) that contains a live *aroA*-deleted *E. coli* strain that showed reduction of incidence of lesions typical of colibacilosis (pericarditis, perihepatitis, airsacculitis) and a significant reduction of mortality due to *E. coli* O78 infections. Another mutant of *E. coli* serovar O78 has been produced using an allelic exchange procedure. Its administration via various routes, such as spray and eye drop for chickens as well as in ovo-inoculation, evoked an effective immune response that protected against a virulent wild-type *E. coli* O78 strain (Nagano et al. 2012).

Freitag et al. (2008) investigated the viability of bacteriophage therapy to combat canine and feline *E. coli* UTIs by testing the activity of naturally occurring bacteriophages on UPEC. They found that most of UPEC are susceptible to bacteriophages, being this a promising strategy as therapeutic agents for treatment of canine and feline *E. coli* UTIs.

References

Alonso M, Sanz M, Irino K, Krüger A, Lucchesi P, Padola N (2016) Isolation of atypical enteropathogenic *Escherichia coli* from chicken and chicken-derived products. Br Poult Sci 57:161–164

Alustiza F, Picco N, Bellingeri R, Terzolo H, Vivas A (2012) Frequency of virulence genes of *Escherichia coli* among newborn piglets from an intensive pig farm in Argentina. Rev Argent Microbiol 44:250–254

Andrade GI, Coura FM, Santos EL, Ferreira MG, Galinari GC, Facury Filho EJ, de Carvalho AU, Lage AP, Heinemann MB (2012) Identification of virulence factors by multiplex PCR in *Escherichia coli* isolated from calves in Minas Gerais, Brazil. Trop Anim Health Prod 44:1783–1790

Aragão AZB, Teocchi MA, Fregolente MCD, Gatti MSV, Pires AV, Yano T (2012) Colibacillosis in lambs is associated to type I heat-stable enterotoxin in a farm in São Paulo State, Brazil. Ciencia Rural 42:854–857

Assumpção GLH, Cardozo MV, Beraldo LG, Maluta RP, Silva JT, de Avila FA, McIntosh D, Rigobelo EC (2015) Antimicrobials resistance patterns and the presence of *stx*1, *stx*2 and *eae* in *Escherichia coli*. Rev Bras Saúde Prod Anim 16:308–316

Bartels CJ, Holzhauer M, Jorritsma R, Swart WA, Lam TJ (2010) Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Prev Vet Med 93:162–169

Bélanger L, Garenaux A, Harel J, Boulianne M, Nadeau E, Dozois CM (2011) *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunol Med Microbiol 62:1–10

Bentancor A (2016) Síndrome urémico hemolítico en áreas urbanas. Rev Argent Microbiol 48:1–4

Bentancor A, Rumi MV, Gentilini MV, Sardoy C, Irino K, Agostini A, Cataldi A (2007) Shiga toxin-producing and attaching and effacing *Escherichia coli* in cats and dogs in a high hemolytic uremic syndrome incidence region in Argentina. FEMS Microbiol Lett 267:251–256

Bentancor A, Vilte DA, Rumi MV, Carbonari CC, Chinen I, Larzabal M, Cataldi A, Mercado EC (2010) Characterization of non-Shiga-toxin-producing *Escherichia coli* O157 strains isolated from dogs. Rev Argent Microbiol 42:46–48

Bentancor AB, Ameal LA, Calviño MF, Martinez MC, Miccio L, Degregorio OJ (2011) Risk factors for Shiga toxin-producing *Escherichia coli* infections in preadolescent school children in Buenos Aires, Argentina. J Infect Dev Ctries 6:378–386

Bicalho R, Machado V, Bicalho M, Gilbert R, Teixeira A, Caixeta L, Pereira R (2010) Molecular and epidemiological characterization of bovine intrauterine *Escherichia coli*. J Dairy Sci 93:5818–5830

Bicalho M, Machado V, Oikonomou G, Gilbert R, Bicalho R (2012) Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. Vet Microbiol 157:125–131

Bidaisee S, Macpherson CN (2014) Zoonoses and one health: a review of the literature. J Parasitol Res 2014:874345

Blanco Crivelli X, Rumi MV, Carfagnini JC, Degregorio O, Bentancor AB (2012) Synanthropic rodents as possible reservoirs of shigatoxigenic *Escherichia coli* strains. Front Cell Infect Microbiol 2:134

Blanco Crivelli X, Llorente P, Bentancor A (2013) Perfil de suceptibilidad de cepas de *Escherichia coli* enteropatógeno aisladas a partir de posibles fuentes de infección para el hombre. Rev Argent Microbiol 43:87

Caballero M, Rivera I, Jara LM, Ulloa-Stanojlovic FM, Shiva C (2015) Isolation and molecular identification of potentially pathogenic *Escherichia coli* and *Campylobacter jejuni* in feral pigeons from an urban area in the city of Lima, Peru. Rev Inst Med Trop Sao Paulo 57:393–396

Callaway T, Edrington T, Loneragan G, Carr M, Nisbet D (2013) Current and near-market intervention strategies for reducing Shiga toxin-producing *Escherichia coli* (STEC) shedding in cattle. Agric Food Anal Bacteriol 3:103–120

Cartes Lillo DI (2014) Caracterización molecular de cepas de *Escherichia coli* aisladas desde muestras de leche provenientes de vacas con mastitis bovina clínica y subclínica. Universidad de Chile, Santiago, Chile

Carvalho VM, Spinola T, Tavolari F, Irino K, Oliveira RM, Ramos MCC (2014) Infecções do trato urinário (ITU) de cães e gatos: etiologia e resistência aos antimicrobianos. Pesqui Vet Bras 34:62–70

Chandran A, Mazumder A (2014) Occurrence of diarrheagenic virulence genes and genetic diversity in *Escherichia coli* isolates from fecal material of various avian hosts in British Columbia, Canada. Appl Environ Microbiol 80:1933–1940

Chew DJ, DiBartola SP, Schenck PA (2011) Cystitis and urethritis: urinary tract infection. In: Canine and feline nephrology and urology, 2nd edn. W.B. Saunders, St. Louis, pp 240–271

Chomel BB (2014) Emerging and re-emerging zoonoses of dogs and cats. Animals 4:434–445

Clements A, Young JC, Constantinou N, Frankel G (2012) Infection strategies of enteric pathogenic *Escherichia coli*. Gut Microbes 3:71–87

Colello R, Cáceres ME, Ruiz MJ, Sanz M, Etcheverría AI, Padola NL (2016) From farm to table: follow-up of Shiga toxin-producing *Escherichia coli* throughout the pork production chain in Argentina. Front Microbiol 7:93

Coura FM, Lage AP, Heinemann MB (2014) *Escherichia coli* pathotypes associated with diarrhea in calves: an update. Pesqui Vet Bras 34:811–818

Coura FM, Freitas MD, Ribeiro J, de Leme RA, de Souza C, Alfieri AA, Facury Filho EJ, de Carvalho AÚ, Silva MX, Lage AP (2015) Longitudinal study of *Salmonella* spp., diarrheagenic *Escherichia coli*, *Rotavirus*, and *Coronavirus* isolated from healthy and diarrheic calves in a Brazilian dairy herd. Trop Anim Health Prod 47:3–11

Cox E, Melkebeek V, Devriendt B, Goddeeris B, Vanrompay D (2014) Vaccines against enteric *E. coli* infections in animals. In: Morabito S (ed) Pathogenic *Escherichia coli*: molecular and cellular microbiology. Caister Academic Press, Norfolk, pp 255–270

Croxen MA, Finlay BB (2010) Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol 8:26–38

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26:822–880

Cruz Junior EC, Salvarani FM, Silva RO, Silva MX, Lobato FC, Guedes R (2013) A surveillance of enteropathogens in piglets from birth to seven days of age in Brazil. Pesqui Vet Bras 33:963–969

Cundon C, Ameal A, Maubecín E, Bentancor A (In Press) Caracterización de cepas de *Escherichia coli* patógeno extraintestinal aislados de perros y gatos de Buenos Aires, Argentina. InVet

Cunha MPV, de Oliveira MGX, de Oliveira MCV, da Silva KC, Gomes CR, Moreno AM, Knöbl T (2014) Virulence profiles, phylogenetic background, and antibiotic resistance of *Escherichia coli* isolated from Turkeys with Airsacculitis. Sci World J 2014:289024

Dogan B, Rishniw M, Bruant G, Harel J, Schukken YH, Simpson KW (2012) Phylogroup and *lpf*A influence epithelial invasion by mastitis associated *Escherichia coli*. Vet Microbiol 159:163–170

Etcheverria AI, Padola NL (2013) Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization. Virulence 4:366–372

Etcheverria A, Arroyo G, Perdigon G, Parma A (2006) *Escherichia coli* with anti-O157: H7 activity isolated from bovine colon. J Appl Microbiol 100:384–389

Fecteau G, Smith BP, George LW (2009) Septicemia and meningitis in the newborn calf. Vet Clin North Am Food Anim Pract 25:195–208

Feng PC, Keys C, Lacher DW, Beutin L, Bentancor A, Heuvelink A, Afset JE, Rumi V, Monday S (2012) Clonal relations of atypical enteropathogenic *Escherichia coli* O157:H16 strains isolated from various sources from several countries. FEMS Microbiol Lett 337:126–131

Ferens WA, Hovde CJ (2011) *Escherichia coli* O157:H7: animal reservoir and sources of human infection. Foodborne Pathog Dis 8:465–487

Fernandes JBC, Zanardo LG, Galvão NN, Carvalho IA, Nero LA, Moreira MAS (2011) *Escherichia coli* from clinical mastitis serotypes and virulence factors. J Vet Diagn Invest 23:1146–1152

Fernández D, Irino K, Sanz M, Padola N, Parma A (2010) Characterization of Shiga toxin-producing *Escherichia coli* isolated from dairy cows in Argentina. Lett Appl Microbiol 51:377–382

Fernández D, Sanz ME, Parma AE, Padola NL (2012) Short communication: characterization of Shiga toxin-producing *Escherichia coli* isolated from newborn, milk-fed, and growing calves in Argentina. J Dairy Sci 95:5340–5343

Freitag T, Squires RA, Schmid J (2008) Naturally occurring bacteriophages lyse a large proportion of canine and feline uropathogenic *Escherichia coli* isolates *in vitro*. Res Vet Sci 85:1–7

Freitas Filho EG, Ferreira MR, Pinto JF, Conceição FR, Moreira CN (2014) Enterohemorrhagic *Escherichia coli* O157:H7 from healthy dairy cattle in Mid-West Brazil: occurrence and molecular characterization. Pesqui Vet Bras 34:24–28

Gentilini MB, Molina LR, Facury Filho EJ, Moreira GHFA, Moreira LPV, Gonçalves RL, de Carvalho AU (2013) *Escherichia coli* J5: immunization of dairy cows against *Escherichia coli* mastitis. Ciencia Rural 43:1843–1851

Gill A, Gill CO (2010) Non-O157 verotoxigenic *Escherichia coli* and beef: a Canadian perspective. Can J Vet Res 74:161–169

Gyles CL, Fairbrother J (2010) *Escherichia coli*. In: Gyles CL, Prescott JF, Songer JG, Thoen CO (eds) Pathogenesis of bacterial infections in animals. Wiley, Ames, pp 267–308

Hebbelstrup Jensen B, Olsen KE, Struve C, Krogfelt KA, Petersen AM (2014) Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev 27:614–630

Hurd HS, Malladi S (2012) An outcomes model to evaluate risks and benefits of *Escherichia coli* vaccination in beef cattle. Foodborne Pathog Dis 9:952–961

Hussain T (2015) An introduction to the serotypes, pathotypes and phylotypes of *Escherichia coli*. Int J Microbiol Allied Sci 2:9–16

Jay-Russell MT, Hake AF, Bengson Y, Thiptara A, Nguyen T (2014) Prevalence and characterization of *Escherichia coli* and *Salmonella* strains isolated from stray dog and coyote feces in a major leafy greens production region at the United States-Mexico border. PLoS One 9, e113433

Kassé F, Fairbrother J, Dubuc J (2016) Relationship between *Escherichia coli* virulence factors and postpartum metritis in dairy cows. J Dairy Sci 99:4656–4667

Kempf F, Slugocki C, Blum SE, Leitner G, Germon P (2016) Genomic comparative study of bovine mastitis *Escherichia coli*. PLoS One 11, e0147954

Kolenda R, Burdukiewicz M, Schierack P (2015) A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. coli*. Front Cell Infect Microbiol 5:23

Krause DO, Bhandari SK, House JD, Nyachoti CM (2010) Response of nursery pigs to a synbiotic preparation of starch and an anti-*Escherichia coli* K88 probiotic. Appl Environ Microbiol 76:8192–8200

Krüger A, Lucchesi PM, Parma AE (2011) Verotoxins in bovine and meat verotoxin-producing *Escherichia coli* isolates: type, number of variants, and relationship to cytotoxicity. Appl Environ Microbiol 77:73–79

Krüger A, Lucchesi PM, Sanso AM, Etcheverria AI, Bustamante AV, Burgan J, Fernandez L, Fernandez D, Leotta G, Friedrich AW, Padola NL, Rossen JW (2015) Genetic characterization of Shiga toxin-producing *Escherichia coli* O26:H11 strains isolated from animal, food, and clinical samples. Front Cell Infect Microbiol 5:74

Kunert Filho H, Brito K, Cavalli L, Brito B (2015) Avian pathogenic *Escherichia coli* (APEC)-an update on the control. In: Méndez-Vilas A (ed) The battle against microbial pathogens: basic science, technological advances and educational programs, vol 2, 5th edn, Microbiology. Formatex Research Center, Badajoz, pp 598–618

Liu X, Thungrat K, Boothe DM (2015) Multilocus sequence typing and virulence profiles in uropathogenic *Escherichia coli* isolated from cats in the United States. PLoS One 10, e0143335

López O, Duverne L, Chinen I, Carbonari C, Mazieres J, Deza N, Baschier A, Miliwebski E, Ibelli V, Shicariol Pinheiro S, Rivas M (2012) Shedding and characterization non-O157 of Shiga toxin-producing *Escherichia coli* strains isolated from beef cattle in one feedlot of Argentina. In: VTEC(2012) eighth international symposiumon shigatoxin (verocytotoxin) producing *Escherichia coli* infections, 2012

Luna E, Maturrano H, Rivera G, Zanabria H, Rosadio A (2012) Genotipificación, evaluación toxigénica *in vitro* y sensibilidad a antibióticos de cepas de *Escherichia coli* aisladas de casos diarreicos y fatales en alpacas neonatas. Rev Investig Vet Peru 23:280–288

Lyhs U, Ikonen I, Pohjanvirta T, Raninen K, Perko-Mäkelä P, Pelkonen S (2012) Extraintestinal pathogenic *Escherichia coli* in poultry meat products on the Finnish retail market. Acta Vet Scand 54:1–6

Maluta RP, Fairbrother JM, Stella AE, Rigobelo EC, Martinez R, de Ávila FA (2014) Potentially pathogenic *Escherichia coli* in healthy, pasture-raised sheep on farms and at the abattoir in Brazil. Vet Microbiol 169:89–95

Masana M, D'Astek B, Palladino P, Galli L, Del Castillo L, Carbonari C, Leotta G, Vilacoba E, Irino K, Rivas M (2011) Genotypic characterization of non-O157 Shiga toxin–producing *Escherichia coli* in beef abattoirs of Argentina. J Food Prot 74:2008–2017

Mellata M (2013) Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. Foodborne Pathog Dis 10:916–932

Miko A, Rivas M, Bentancor A, Delannoy S, Fach P, Beutin L (2014) Emerging types of Shiga toxin-producing *E. coli* (STEC) O178 present in cattle, deer, and humans from Argentina and Germany. Front Cell Infect Microbiol 4:78

Moredo FA, Pineyro PE, Márquez GC, Sanz M, Colello R, Etcheverría A, Padola NL, Quiroga MA, Perfumo CJ, Galli L (2015) Enterotoxigenic *Escherichia coli* subclinical infection in pigs: bacteriological and genotypic characterization and antimicrobial resistance profiles. Foodborne Pathog Dis 12:704–711

Nagano T, Kitahara R, Nagai S (2012) An attenuated mutant of avian pathogenic *Escherichia coli* serovar O78: a possible live vaccine strain for prevention of avian colibacillosis. Microbiol Immunol 56:605–612

Okhuysen PC, DuPont HL (2010) Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. J Infect Dis 202:503–505

Orden JA, Dominguez-Bernal G, Martinez-Pulgarin S, Blanco M, Blanco JE, Mora A, Blanco J, Blanco J, de la Fuente R (2007) Necrotoxigenic *Escherichia coli* from sheep and goats produce a new type of cytotoxic necrotizing factor (CNF3) associated with the *eae* and *ehx*A genes. Int Microbiol 10:47–55

Osugui L, de Castro AP, Iovine R, Irino K, Carvalho V (2014) Virulence genotypes, antibiotic resistance and the phylogenetic background of extraintestinal pathogenic *Escherichia coli* isolated from urinary tract infections of dogs and cats in Brazil. Vet Microbiol 171:242–247

Padola NL, Etcheverría AI (2014) Shiga toxin-producing *Escherichia coli* in human, cattle, and foods. Strategies for detection and control. Front Cell Infect Microbiol 4:89

Persad AK, LeJeune JT (2014) Animal reservoirs of Shiga toxin-producing *Escherichia coli*. Microbiol Spectr 2:EHEC-0027-2014

Picco NY, Alustiza FE, Bellingeri RV, Grosso MC, Motta CE, Larriestra AJ, Vissio C, Tiranti KI, Terzolo HR, Moreira AR (2015) Molecular screening of pathogenic *Escherichia coli* strains isolated from dairy neonatal calves in Cordoba province, Argentina. Rev Argent Microbiol 47:95–102

Prioste FES, Cunha MPV, Teixeira RHF, Zwargg T, Di-Chiacchio RG, Melville PA, Benites NR, Sinhorini J, Matushima ER, Knöbl T (2013) Similaridade genética entre APEC e cepas de *Escherichia coli* isoladas de *Guaruba guarouba* em um estudo com psitacídeos hígidos de cativeiro. Braz J Vet Res Anim Sci 50:145–151

Puño-Sarmiento J, Medeiros L, Chiconi C, Martins F, Pelayo J, Rocha S, Blanco J, Blanco M, Zanutto M, Kobayashi R (2013) Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. Vet Microbiol 166:676–680

Rabinovitz B, Gerhardt E, Tironi Farinati C, Abdala A, Galarza R, Vilte D, Ibarra C, Cataldi A, Mercado EC (2012) Vaccination of pregnant cows with EspA, EspB, -intimin, and Shiga toxin 2 proteins from *Escherichia coli* O157:H7 induces high levels of specific colostral antibodies that are transferred to newborn calves. J Dairy Sci 95:3318–3326

Ricke SC, Hererra P, Biswas D (2012) Bacteriophages for potential food safety applications in organic meat production. In: Organic meat production and processing. Wiley-Blackwell, Oxford, pp 407–424

Rivera F, Sotelo E, Morales I, Menacho F, Medina A, Evaristo R, Valencia R, Carbajal L, Ruiz J, Ochoa T (2012) Short communication: detection of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle and pigs in Lima, Peru. J Dairy Sci 95:1166–1169

Rodriguez Palacios A, Staempfli H, Duffield T, Weese J (2009) Isolation of bovine intestinal *Lactobacillus plantarum* and *Pediococcus acidilactici* with inhibitory activity against *Escherichia coli* O157 and F5. J Appl Microbiol 106:393–401

Roug A, Byrne BA, Conrad PA, Miller WA (2013) Zoonotic fecal pathogens and antimicrobial resistance in county fair animals. Comp Immunol Microbiol Infect Dis 36:303–308

Rozema EA, Stephens TP, Bach SJ, Okine EK, Johnson RP, Stanford K, McAllister TA (2009) Oral and rectal administration of bacteriophages for control of *Escherichia coli* O157:H7 in feedlot cattle. J Food Prot 72:241–250

Rumi MV, Irino K, Deza N, Huguet MJ, Bentancor AB (2012) First isolation in Argentina of a highly virulent Shiga toxin-producing *Escherichia coli* O145:NM from a domestic cat. J Infect Dev Ctries 6:358–363

Santona S, Diaz N, Fiori PL, Francisco M, Sidat M, Cappuccinelli P, Rappelli P (2013) Genotypic and phenotypic features of enteropathogenic *Escherichia coli* isolated in industrialized and developing countries. J Infect Dev Ctries 7:214–219

Sargeant J, Amezcua M, Rajic A, Waddell L (2007) Pre-harvest interventions to reduce the shedding of *E. coli* O157 in the faeces of weaned domestic ruminants: a systematic review. Zoonoses Public Health 54:260–277

Saviolli JY, Cunha MPV, Guerra MFL, Irino K, Catão-Dias JL, de Carvalho VM (2016) Free-ranging frigates (*Fregata magnificens*) of the southeast coast of Brazil harbor extraintestinal pathogenic *Escherichia coli* resistant to antimicrobials. PLoS One 11, e0148624

Servin AL (2014) Pathogenesis of human diffusely adhering *Escherichia coli* expressing Afa/Dr adhesins (Afa/Dr DAEC): current insights and future challenges. Clin Microbiol Rev 27:823–869

Shpigel NY, Elazar S, Rosenshine I (2008) Mammary pathogenic *Escherichia coli*. Curr Opin Microbiol 11:60–65

Silva VL, Nicoli JR, Nascimento TC, Diniz CG (2009) Diarrheagenic *Escherichia coli* strains recovered from urban pigeons (*Columba livia*) in Brazil and their antimicrobial susceptibility patterns. Curr Microbiol 59:302–308

Silvera E, Perales R, Rodríguez J, López T, Gavidia C, Agapito J, Palacios C (2012) Presencia de *Escherichia coli* O157 en crías de alpacas (*Vicugna pacos*). Rev Investig Vet Peru 23:98–104

Snedeker K, Campbell M, Sargeant J (2012) A systematic review of vaccinations to reduce the shedding of *Escherichia coli* O157 in the faeces of domestic ruminants. Zoonoses Public Health 59:126–138

Trotz-Williams LA, Mercer NJ, Walters JM, Maki AM, Johnson RP (2012) Pork implicated in a Shiga toxin-producing *Escherichia coli* O157:H7 outbreak in Ontario, Canada. Can J Public Health 103:e322–e326

Uber AP, Trabulsi LR, Irino K, Beutin L, Ghilardi ÅC, Gomes TA, Liberatore AMA, de Castro AF, Elias WP (2006) Enteroaggregative *Escherichia coli* from humans and animals differ in major phenotypical traits and virulence genes. FEMS Microbiol Lett 256:251–257

Vilte D, Larzábal M, Mayr U, Garbaccio S, Gammella M, Rabinovitz B, Delgado F, Meikle V, Cantet R, Lubitz P (2012) A systemic vaccine based on *Escherichia coli* O157:H7 bacterial ghosts (BGs) reduces the excretion of *E. coli* O157:H7 in calves. Vet Immunol Immunopathol 146:169–176

Wagener K, Grunert T, Prunner I, Ehling-Schulz M, Drillich M (2014) Dynamics of uterine infections with *Escherichia coli*, *Streptococcus uberis* and *Trueperella pyogenes* in post-partum dairy cows and their association with clinical endometritis. Vet J 202:527–532

Wells JE, Bartges JW, Kania SA, Bemis DA, Gluhak T (2013) Association between presence of urovirulence factors, phylogenetic class, and antimicrobial resistance patterns in 159 uropathogenic *Escherichia coli* samples isolated from dogs. Open J Vet Med 3:193–203

Wisener L, Sargeant J, O'Connor A, Faires M, Glass Kaastra S (2015) The use of direct-fed microbials to reduce shedding of *Escherichia coli* O157 in beef cattle: a systematic review and meta-analysis. Zoonoses Public Health 62:75–89

Wong C, Epstein S, Westropp J (2015) Antimicrobial susceptibility patterns in urinary tract infections in dogs (2010–2013). J Vet Intern Med 29:1045–1052

Zhang W (2014) Progress and challenges in vaccine development against enterotoxigenic *Escherichia coli* (ETEC)-associated porcine Post-Weaning Diarrhoea (PWD). J Vet Med Res 1(2):1006

Zuraw L (2013) Study: *E. coli* cattle vaccination could prevent 83 percent of human cases. Food Safety News, 23 Sept 2013

Chapter 8
Escherichia coli in Food Products

Lucía Galli, Victoria Brusa, Ricardo Rodríguez, Marcelo Signorini, Juan M. Oteiza, and Gerardo A. Leotta

Summary Foodborne diseases, as a result of the consumption of food contaminated by diarrheagenic *E. coli* (DEC), have been recognized as one of the most prevalent health issues worldwide. Certain pathogroups are typically transmitted by contaminated food and water; however, their prevalence in food is restricted to outbreaks and research studies in certain regions. These bacteria are affected by a multiplicity of limiting factors present in food, e.g., temperature, pH, water activity, food processing, and intrinsic microorganism factors such as injury and inoculum. Emerging intervention processing techniques are receiving good attention because of their potential for food quality and safety improvement. During the last decade, some of these technologies—high-pressure processing, high-pressure homogenization, pulsed electric field, ultraviolet light, intense light pulses, ultrasound, radiation, ozone and organic acids, among others—have been tested to control DEC in food. Food safety must be principally ensured by a more

L. Galli • V. Brusa • G.A. Leotta (✉)
Facultad de Ciencias Veterinarias, IGEVET—Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET LA PLATA), Universidad Nacional de La Plata, La Plata, Argentina
e-mail: luciagalli@hotmail.com; toibrusa@hotmail.com; gerardo.leotta@gmail.com

R. Rodríguez
Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingam, Argentina
e-mail: rodriguez.ricardo@inta.gob.ar

M. Signorini
CONICET—Estación Experimental Agropecuaria INTA, Rafaela, Argentina
e-mail: marcelo.signorini@gmail.com

J.M. Oteiza
Laboratorio de Microbiología de los Alimentos, Centro de Investigación y Asistencia Técnica a la Industria (CIATI AC)-CONICET, Centenario, Argentina
e-mail: juano@ciati.com.ar

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_8

preventative approach, such as product and process design and the application of the Good Hygiene and Manufacturing Practices and the Hazard Analysis Critical Control Point principles. Epidemiological studies provide invaluable information to define more effective management strategies. In this context, risk analysis tools have proven effectiveness to reduce foodborne diseases through the design, development, implementation, evaluation, and communication of control measures to protect the public health. To achieve a strategic control of *E. coli* infections, a multidisciplinary approach through stages of the agro-food chain is required to generate evidence-based risk management measures. Only in this way will it be possible to protect the health of consumers.

1 General Aspects

Food security exists when all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life (FAO 2001). Food safety refers to all those hazards, whether chronic or acute, that may make food injurious to the health of the consumer. Quality includes all attributes that influence a product's value to the consumer, including negative attributes such as spoilage, contamination with filth, discoloration, off-odors, and positive attributes such as the origin, color, flavor, texture, and processing method of the food (FAO/WHO 2003). It is well known that the best alternative to minimize microbial growth and loss of food quality during processing and storage is the combined action of limiting factors of microbial growth. This approach allows the harmonization of better food sensory quality with safety and supports new trends in food production and food processing.

Food production is evolving toward the integration of local production and industrial exportation, including quality assurance systems. Ensuring the microbiological quality of food is one of the aspects related to public health. In this sense, the One Health Initiative is interesting because it involves human, animal, and environmental health (http://www.onehealthinitiative.com). Food safety and quality are essential in the food chain production. To accomplish these premises in a strategic way, a multidisciplinary approach is required to generate evidence-based risk management measures.

We consider it essential for diseases caused by DEC to start by addressing the agro-food chain concept based on foodborne disease epidemiology. In this way, we can go into more depth about concepts related to DEC pathogroups, DEC and food microbial ecology, and the intervention strategies to reduce *E. coli* in food. Second, we summarize the specific regulations for DEC in America, including microbiological criteria and official methodologies to detect and isolate DEC. Finally, we integrate the control of *E. coli* foodborne disease determinants, considering quantitative risk assessment.

2 Epidemiological Tools

Contaminated food is a major health concern, especially in developing countries. While it is difficult to assess the incidence of foodborne disease, different estimates consider that at least one-third of the population is affected annually in developed countries. However, as not all the cases are reported to the epidemiological surveillance systems, the real incidence is undoubtedly higher. In developing countries, the problem is even worse, because foodborne disease data are rarely available (Cherry et al. 2014).

Foodborne diseases are caused by the consumption of food contaminated with *E. coli* and have been recognized among the most prevalent worldwide (Cherry et al. 2014; Majowicz et al. 2014). Considering their significant impact on public health, it is necessary to identify and adopt risk management measures to reduce the incidence rate. However, such identification should take into account the scientific information available so that risk managers can be sure about the efficacy of the adopted measures.

Epidemiology is the science that can provide valuable information to define the most appropriate risk management measurement to reduce the exposure to foodborne pathogens (Silman and Macfarlane 2002). Epidemiologists try to identify the factors that increase the probability to get sick. Once these factors are identified, it is possible to design targeted intervention strategies. Sources of information for this epidemiological approach include (a) epidemiologic surveillance systems, (b) outbreak studies, and (c) quantitative risk assessment.

2.1 Epidemiologic Surveillance

Epidemiologic surveillance collects information used for planning, implementing, and evaluating public health interventions and programs. Surveillance data are used both to determine the need for public health action and to assess the effectiveness of programs (Ammon and Makela 2010). Traditionally, reportable disease data have been the main source of information to describe the burden of specific pathogenic causes of diarrheal disease in the population. However, such data capture only a fraction of the true number of cases. Most American countries do not have reliable data on the occurrence of diarrhea caused by *E. coli*. This is because epidemiological surveillance systems are not sufficiently consolidated and, if present, surveillance is syndrome specific (e.g., diarrheal diseases) and it is rarely possible to determine the etiological agent involved in clinical cases. Thus, it is difficult to obtain updated and reliable data on the incidence of *E. coli* diarrheal cases, and even more difficult to estimate the prevalence of different *E. coli* groups. However, some American countries have consolidated epidemiologic surveillance systems from which it is possible to estimate the impact of *E. coli* diarrhea.

Shiga toxin-producing *E. coli* (STEC) strains can cause different illnesses. The Public Health Agency of Canada registers Canadian STEC infections through the National Notifiable Database. Using these data, the incidence rate of STEC infection

was estimated in four cases/100,000 populations per year (Thomas et al. 2006). However, the true incidence rate is likely to be 10–47 times greater because most STEC infection cases are not reported (Smith et al. 2013).

An estimated 63,153 *E. coli* O157:H7 infections occur in the United States annually, resulting in an estimated 2138 hospitalizations and 20 deaths (Painter et al. 2013). According to the Centers for Disease Control and Prevention (CDC), the incidence of *E. coli* O157:H7 infection is approximately 1.06 cases/100,000 population.

In Argentina, the Epidemiologic Surveillance System of Communicable Diseases is mostly syndrome specific (Boletín Integrado de Epidemiología 298). For that reason, only the incidence of diarrhea is informed, but data about the etiologic agent are scarce. However, a specific surveillance system collects information from Sentinel Units which record hemolytic uremic syndrome (HUS) cases. In Argentina, HUS is the principal pediatric cause of acute renal failure, the second cause of chronic renal failure, and is responsible for 20 % of kidney transplants in children and adolescents (Rivas et al. 2011). Annually, between 300 and 500 new HUS cases are reported, with an average annual incidence rate of 1.1 cases/100,000 inhabitants and 8.4 cases/100,000 children under 5 years of age (Boletín Integrado de Epidemiología 298).

In view of the absence of epidemiologic surveillance systems, Majowicz et al. (2014) calculated the global annual number of STEC infections resulting in HUS cases. In America, the estimated STEC infection incidence rate was approximately 58.7 cases/100,000 inhabitants, and the estimated HUS incidence rate was 0.17 cases/100,000 inhabitants. Although these data are underreported and have some limitations, the study provided a global estimate of the impact of STEC infection on public health.

2.2 Outbreak Studies

Epidemiological reports of foodborne outbreaks are mostly case–control or retrospective cohort studies. In the case of STEC infection, cases are identified together with patients without the disease (controls). Cases and controls are then compared to identify the source of infection, considering their exposure to different foods (Silman and Macfarlane 2002). These studies might be complex to perform for various reasons, namely, undefined cohorts, small number of cases, difficulty to find controls comparable with cases, and susceptibility to biases (recall bias) (Gaulin et al. 2012).

The prevention of *E. coli* infections is challenging because resources are limited and linking individual illnesses to a particular food is rarely possible, except during an outbreak (Painter et al. 2013). To prevent foodborne illnesses, it is crucial to prioritize the limited food safety resources across a large number of foods. In this sense, outbreak studies are the only way to associate a foodborne disease with a particular food, and results could help to establish a rank of foods mostly implicated in the transmission of *E. coli*. However, most American countries lack specific

resources to conduct case–control studies during an outbreak and, consequently, they are not able to define a food management strategy to reduce the impact of foodborne diseases.

In the period 1998–2008, 206 *E. coli* outbreaks were reported in the United States, 186 of which were due to *E. coli* O157:H7. Transmission was through the consumption of meat (33.0–41.3 %), leafy vegetables (19.3–31.5 %), and dairy products (6.7–9.8 %). Interestingly, the foods identified in outbreaks of *E. coli* non-O157 (six in the same period) were fruits and beef (Painter et al. 2013). Another study evaluating *E. coli* O157:H7 outbreaks in the US during 2003–2012 arrived at similar conclusions. Beef, leafy vegetables, and dairy products (milk and cheese) were the foods associated with the transmission (Heiman et al. 2015). The proportion of female patients was higher in outbreaks attributed to fruits (67 %) and leaf vegetables (65 %), and lower in those attributed to meats other than beef (31 %). *E. coli* infections were evenly distributed among women and men in outbreaks attributed to beef and dairy. These data would indicate a gender-specific food preference and, consequently, a differential risk of exposure (Heiman et al. 2015).

Beef, particularly ground beef, continues to be the major source of *E. coli* O157 outbreaks (Torso et al. 2015), likely because cattle are the main reservoir for this pathogen. However, outbreaks attributed to leafy vegetables, dairy products, fruits, and other meats were more severe than outbreaks attributed to beef, probably due to a change in strain virulence and host susceptibility by patient age and sex (Heiman et al. 2015).

Although *E. coli* O157 outbreaks occur throughout the year, most of them are reported during summer. However, such seasonality varies by food category. Beef-associated outbreaks occur mostly in summer and leafy vegetable-associated outbreaks during fall. Cattle shed the largest number of *E. coli* O157 organisms in their feces during the summer months, coinciding with a higher prevalence of *E. coli* O157 on hides in processing plants. On the other hand, leafy vegetable-associated outbreaks exhibit another pattern of presentation, which could be the result of summertime applications to seedlings of irrigation water, soil amendments, or fertilizers that might contain more *E. coli* O157:H7 organisms than in other seasons (Heiman et al. 2015). Additionally, washing prewashed spinach before consumption might not decrease the risk of illness, because *E. coli* O157:H7 can persist on vegetables for long periods after waterborne contamination, and the available sanitation methods are not completely effective at removing organisms (Wendel et al. 2009).

In Argentina, although the magnitude of outbreaks has been lower compared with the United States (Torso et al. 2015; Wendel et al. 2009) and Canada (Gaulin et al. 2012), the national surveillance system has reported the appearance of sporadic numerous cases (Rivas et al. 2011). *E. coli* O157:H7 was the strain most commonly isolated from human HUS cases (74.6 % of the noticeable cases). The frequency of non-O157 *E. coli* isolation was as follows: O145 [H27; H–; NT] (13.6 %), O121 [H19] (2.2 %), O26 [H2;11; NT] (1.4 %), O174 [H8; 21; 28; H–] (1.0 %); other serotypes, 7.2 %.

The improvement of national epidemiologic surveillance systems and outbreak studies enhance the understanding of the epidemiology of *E. coli* infections and help national health systems to monitor appropriately changes in the frequency of

the main serogroups, which cause diseases over time (Brooks et al. 2005). Strengthening public health systems to prevent and control foodborne diseases requires timely reporting of suspected and confirmed *E. coli* cases. Further, it requires high indices of suspicion for foodborne diseases, accurate diagnosis, and commitment to maximize the available information through timely application of molecular subtyping (Wendel et al. 2009).

3 Diarrheagenic *E. coli* Pathogroups in Food

DEC strains are an important cause of intestinal disease in the developing world. They mainly affect children and are now recognized as emerging enteropathogens in the developed world. Certain DEC pathogroups are transmitted typically by contaminated food and water; however, their prevalence in food items that are produced, consumed, and sometimes exported worldwide has not been well investigated. This diagnosis would provide epidemiologically important information, especially with respect to routes of spread. Nevertheless, the current methods of detection are expensive and labor intensive for routine detection.

In Mexico, 5162 food items and beverages consumed throughout the Sinaloa state were tested for their microbiological quality. The prevalence of DEC strains was low (1.08 %), with dairy products being the most contaminated food items (2.8 %), followed by meat derivatives, seafood, and fish. On the other hand, DEC strains were not present in beverages and ice samples. Among the DEC pathogroups detected in food items, EPEC was the most prevalent (78.5 %), followed by EAEC (10.7 %), STEC (8.9 %), and ETEC (1.7 %) (Canizalez-Roman et al. 2013).

In Colombia, food product samples consisting of pasteurized milk, unpasteurized fruit juice, ground beef, cheese, and vegetables obtained at four retail stores were also analyzed for their microbiological quality. The prevalence of intestinal *E. coli* contamination was low (2.1 %); ground beef and cheese samples were the only contaminated food items, being EPEC, ETEC, and STEC the DEC pathogroups detected (Amézquita-Montes et al. 2015).

Water could be one of the principal vehicles of food contamination. In Peru, DEC strains were found in 33 % of the drinking water samples analyzed in rural areas (Gil et al. 2014).

Unpasteurized juice has been associated with foodborne outbreaks for many years. In Mexico, microbiological quality analyzed in fresh beetroot and carrot juice samples was poor (Gómez-Aldapa et al. 2014). DEC strains were detected in 9 and 8.9 % of the samples analyzed, respectively. ETEC, EIEC, aEPEC, STEC were among the DEC identified and, in some samples, two different pathogroups were simultaneously isolated. Some critical points identified in contamination included no washing and/or disinfection in raw materials, cross contamination during juice preparation, and bad storage conditions.

The consumption of ready-to-eat (RTE) salads has increased worldwide, with the concomitant increase in the number of outbreaks caused by food-borne pathogens, including DEC. In a Mexican study, among the 130 salad samples analyzed, 6.2 %

were contaminated with DEC strains, mainly EIEC, ETEC, and non-O157 STEC strains (Castro-Rosas et al. 2012). In another recent study, DEC strains (STEC, EPEC, and ETEC pathogroups) were identified in 8 % of nopalitos (tender cactus) salad samples tested. Interestingly, all isolated strains exhibited multidrug resistance, demonstrating for the first time this kind of profiles in the samples analyzed (Gómez-Aldapa et al. 2016).

Other studies focused their attention on fresh produce usually consumed as RTE (coriander, parsley, spinach, lettuce, alfalfa sprouts, and tomatoes). The authors described the presence of ETEC strains in tomatoes, alfalfa sprouts, coriander, and parsley, with prevalence rates of approximately 4 % through 0.3 % (Feng and Reddy 2014; Gómez-Aldapa et al. 2013; Rangel-Vargas et al. 2015). STEC strains were detected in tomatoes, alfalfa sprouts, and spinach, with prevalence rates of 6 % through 0.5 % (Feng and Reddy 2014; Gómez-Aldapa et al. 2013; Rangel-Vargas et al. 2015). About 9 % of the spinach isolates presented STEC O157:H7 and O26:H11. *E. coli* O157:H7 was not detected in any STEC-positive samples from tomato and alfalfa sprouts. EPEC strains were found in tomatoes and alfalfa sprouts, with prevalence rates of approximately 4 % (Gómez-Aldapa et al. 2013; Rangel-Vargas et al. 2015). EIEC strains were isolated in 1 % of saladette-tomato samples (Gómez-Aldapa et al. 2013).

Meats of animal origin could potentially act as transmission vehicles for STEC and other DEC strains. In a study conducted in the USA in *E. coli* isolates collected from 2002 to 2007 under the national retail meat program resistance monitoring system, 17 STEC (16 from ground beef, 1 from pork chop) and 11 aEPEC (5 from chicken breast, 4 from ground beef, 2 from pork chop) strains were detected (Xia et al. 2010). In Canada, following a similar program, 17 aEPEC strains were identified; six were coclassified as aEPEC-ETEC and two as aEPEC-ExPEC (Comery et al. 2013). This study emphasized the high degree of *E. coli* genome plasticity and ongoing evolution that could give rise to novel *E. coli* pathogens.

Studies of STEC prevalence in minced beef samples performed in Argentina by Brusa et al. (2013) found STEC O157:H7 isolated from 12.2 % of raw ground beef samples and 1.7 % of butcher environmental samples, whereas STEC non-O157 was isolated from 14.4 % of raw ground beef samples and 6.7 % of environmental samples. In another study, 8 % of ground beef samples analyzed from butchers located in Buenos Aires was EPEC-positive (Srednik et al. 2014). Hamburgers have also been implicated in numerous outbreaks. In an Argentinean survey of poultry shops, the proportion of EPEC-positive samples among chicken hamburgers was greater than that of STEC-positive ones, while in butcheries, STEC was predominant (Alonso et al. 2012).

In Brazil, the occurrence of DEC strains was investigated in raw kibbeh and chilled shrimp samples. From 70 raw kibbeh samples analyzed, two strains belonging to the O125:H19 and O149:H8 serotypes were positive for the stx_{1c} genetic sequence (Peresi et al. 2016), whereas the EPEC and ETEC pathogroups were detected on the surface of two chilled shrimp samples (Barbosa et al. 2016).

4 Diarrheagenic *E. coli* and Food Microbial Ecology

Knowledge of the ability of DEC to make its way into and persist in foods and understanding of its behavior will result in better control interventions. DEC is differentially affected by a multiplicity of limiting factors present in food, e.g. temperature (T°C), pH, water activity (a_w), food processing (preservatives, dehydration, cooking), and intrinsic microorganism factors (injury and inoculum). In this context, microbial ecology of food can be defined as the study of the interaction among chemical, physical, and structural attributes of food, factors, and process technologies and the corresponding microbiota that constitute the microbial population, in this case DEC organisms (Fig. 8.1) (Rodríguez 2006). Based on the frequency and severity of foodborne illnesses, most of the information available on DEC food microbial ecology refers to STEC as the most significant pathogroup causing foodborne diseases.

4.1 Food Processing Factors Affecting DEC

The main factors affecting the development and survival of microorganisms in food are listed in Table 8.1. They were classified based on a modification of the classical categorization of Mossel (IFT 2002) and adapted to include emerging food preservation processes. Most of these factors interfere with the stability of the cell internal

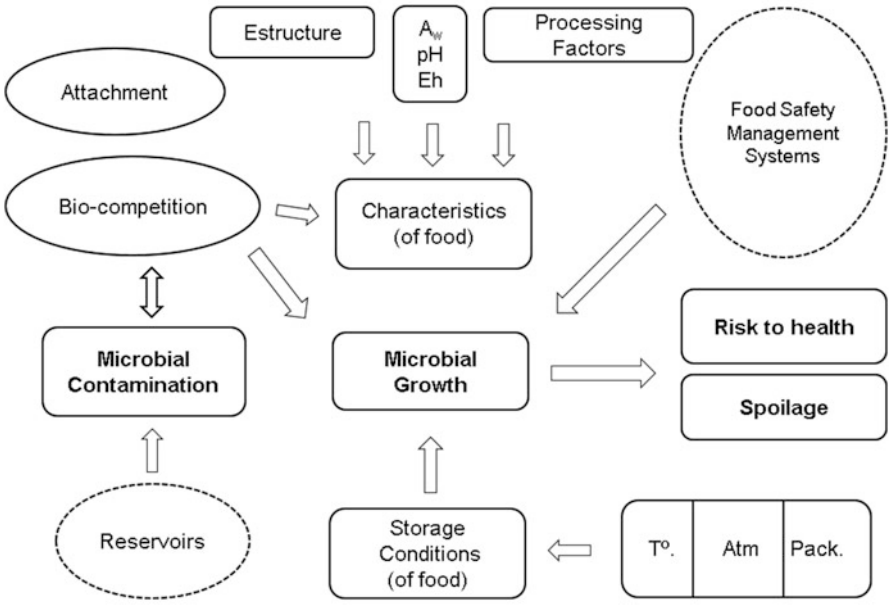

Fig. 8.1 Food microbial ecology

Table 8.1 Main factors involved in food microbial ecology

Type	Main factors
Intrinsic	pH
	Water activity
	Redox potential
	Nutrients
	Viscosity
	Microstructure
	Natural antimicrobials
Processing	Temperature(Pasteurization, sterilization)
	Ionizing radiation
	High hydrostatic pressure (HHP)
	Antimicrobial additives (organic acid, nitrites, sorbates)
	Packaging (modified atmospheres, vacuum)
Extrinsic	Storage temperature (chilling, freezing)
	Environmental gaseous atmosphere
	Environmental humidity
Implicit	Microorganism (physiology, injury)
	Natural microbiota (competition, synergism)

environment of the microorganism represented by variables such as intracellular pH, osmolarity, DNA integrity, and cell membranes. When these interferences occur in a limited range of internal physiological variables, energy-dependent homeostatic mechanisms are triggered in vegetative cells that attempt to restore normal physiological values. This is of fundamental importance in the design of safe food preservation processes, cleaning programs, and sanitation of surfaces and equipment used in the food industry. In terms of DEC organisms, as any other bacteria present in food, pH, a_w, and $T°$ are the three main factors that affect microbial growth (Aertsen and Michiels 2004).

Studies on the thermal sensitivity of *E. coli* O157:H7 in ground beef showed that the pathogen does not have an unusual heat resistance. However, the presence of fat protects the bacteria (Line et al. 1991). D values (time required for a 1-log reduction for a microorganism) at 57.2, 60, 62.8, and 64.3 °C of 270, 45, 24, and 9.6 s were found, respectively. D values for lean (2.0 % fat) and fatty (30.5 % fat) ground beef of 4.1 and 5.3 min at 57.2 °C, respectively, and 0.3 and 0.5 min at 62.8 °C were found, respectively.

Foodborne pathogens must pass through an acidic gastric barrier with pH values as low as 1.5–2.5 to cause infections in humans. These have been studied in STEC, where different mechanisms designated as acid tolerance and acid resistance have been determined (Montville and Matthews 2013). Induction of acid resistance in *E. coli* can also increase tolerance to other environmental stresses, such as heat, radiation, and antimicrobials agents.

The a_w value for total bacterial growth inhibition depends on the availability of free water by cellular enzymes, the presence of defense mechanisms, as well as the solute employed to reduce a_w. Whereas bacteria stop developing at a_w values of 0.90, DEC does at 0.95. The most direct way to reduce a_w in food is by partial dehydration by drying.

Microorganisms can be damaged or injured by effect of sublethal levels of heat, ionizing radiation, weak acids, and sanitizing agents. Such injury is characterized by a reduced resistance to certain selective agents or increased nutritional requirements of certain agents. Another less widespread aspect related to the physiological state of cells that can contaminate food is the phenomenon called viable nonculturable cells (VNCC) which has been described in DEC. This differentiation of vegetative cells, "dormant" viable cells but not "cultured" by the usual techniques of counting, is a survival strategy of these bacteria. Their morphology changes, rods shrink and become spherical, being very different from the corresponding vegetative cells. They can take anywhere from days and weeks to reach the state of VNCC. These cells can be recognized by staining techniques and metabolic activity in response to certain specific substrates. Moreover, some foodborne pathogens that have been in nutrient culture media can become VNCC when subjected to refrigeration temperatures, certainly having strong implications for the safety of refrigerated food products.

4.2 Biofilm Formation

Microbiologically, biofilm formation is defined as communities of microorganisms growing embedded in an exopolysaccharide matrix and adhered to an inert surface, a living tissue or a food. Biofilms can be found in any ecological niche and are of particular importance for the food industry in general and for perishable foods in particular. In essence, if bacteria are present, any surface that combines abundant moisture and nutrients is liable to the formation of biofilms. The development of genomics and proteomics has enabled to identify genes that are expressed differentially when bacteria are forming biofilms. This opens the possibility of identifying control strategies to biofilms using suppressive substances or enzyme inactivation of molecules that facilitate communication among bacterial communities. Although biofilms may have a single type of microorganism, either a pathogen or a spoiler, several bacterial species are much more commonly present. The different kinds of bacteria forming a biofilm can communicate through a cell–cell signaling mechanism known as quorum sensing.

Biofilm allows bacteria to survive in unpredictable scenarios and under conditions of stress, such as changes in temperature, pH, drying, ultraviolet rays, among others. In their mature state, biofilm cells use energy for exopolysaccharide production, which in turn is used as a nutrient. Biofilm bacteria can be 100 times more resistant to antibiotics and up to 300 times more resistant to some sanitizing agents. DEC adhesion to various surfaces has been extensively demonstrated (Fig. 8.2) (Rodríguez 2006).

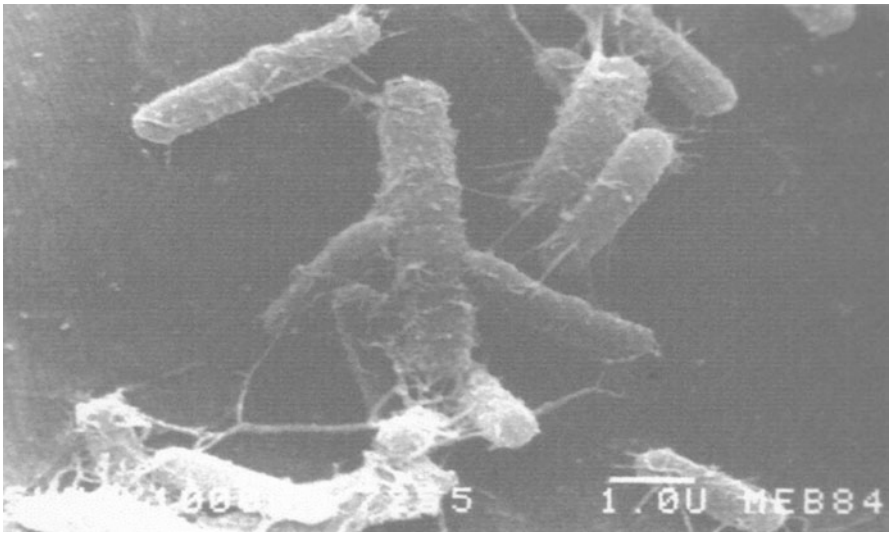

Fig. 8.2 *Escherichia coli* attached to polyethylene film (SEM, ×11,000)

The development of biofilms protects microorganisms and hinders removal of processing equipment, packaging, and food preservation. They are a structured way to provide homeostasis, a real network to develop special functions in cooperation with the cells that form the colonized niche, and finally a great protection against antimicrobial agents. At the level of the industry, it is important to consider the design and engineering of processing equipment and surfaces that are in contact with food to prevent the formation of biofilms by making the initial adsorption process more difficult. Quorum sensing plays a fundamental role in the formation of biofilms of pathogens and spoilage microorganisms; it improves the access to nutrients and micro-favorable niches, providing a unique response that protects against adverse environmental conditions. For this reason, different mechanisms are being investigated and novel compounds to block attachment are being used.

5 Intervention Strategies to Reduce *E. coli* in Food

Emerging processing techniques are receiving good attention because of their potential for food quality and safety improvement. During the last decade, some of these technologies have been tested to control pathogenic *E. coli* in foods because they can inactivate microorganisms at ambient or near ambient temperatures, thus avoiding the deleterious effect of heat on flavor, color, and the nutrient value of foods.

5.1 High-Pressure Processing

It is also called high hydrostatic pressure processing, pascalization, or high-pressure pasteurization. HHP subjects liquid and solid foods, with or without packaging, to pressures between 100 and 800 MPa. Microbial inactivation associated with high-pressure processing (HPP) may be related to one or more factors including cell membrane perturbation, protein denaturation, and biochemical and macromolecular changes (inhibition of DNA, RNA, or protein synthesis). The application of pressures in the range of 300–700 MPa at ambient temperature within a few minutes should result in an inactivation of STEC O157:H7 and non-O157 (including the "big six" serogroups: O103, O111, O26, O145, O121, O45) in different beef types, fruits, drinks, and produce (Zhou et al. 2016).

5.2 High-Pressure Homogenization

It involves the pumping of liquid through a homogenizing valve at high pressure over 100 MPa. It produces high turbulence and shear along with compression, acceleration, and pressure drop, resulting in the breakdown of particles. The effects on bacterial cells are not well known, but the sudden pressure drop probably disrupts microorganisms by torsion and shear stresses, and mostly by cavitation shock waves resulting from imploding gas bubbles. The use of high-pressure homogenization (HPH) was studied by Briñez et al. (2006), who evaluated its effect to reduce *E. coli* O58:H21 in orange juice and *E. coli* O157:H7 in orange juice and whole and skimmed milk.

5.3 Pulsed Electric Field

This technology consists in the application of short duration (1–100 μs) pulses of high voltage (5–80 kV cm^{-1}) to a food placed between two electrodes. Destruction of microbial cells is the result of electroporation of cell membranes. Pulsed electric field (PEF) has been reported to inactivate *E. coli* O157:H7 in different fruits and drinks (Mosqueda-Melgar et al. 2008; Ait-Ouazzou et al. 2013).

5.4 Ultraviolet Light

Treatment with Ultraviolet light (UV) radiation could be an attractive alternative technology to thermal pasteurization that can be applied to inactivate harmful microbes in food. It produces a nonionizing radiation with germicidal properties at

wavelengths in the range of 200–280 nm. UV-C light inactivates microorganisms by damaging their DNA due to the dimerization of thymine bases. Different works have been recently conducted to analyze the effect of UV on STEC O157:H7 and non-O157 in different fruits, drinks, and produce (Oteiza et al. 2010).

5.5 Intense Light Pulses

It is a nonthermal method for food preservation that involves the use of intense and short duration pulses of a broad spectrum to ensure microbial decontamination. Intense light pulses (IPL) employ a flash lamp filled with inert gas, such as xenon, that emits high-frequency pulses of broad-spectrum radiation containing wavelengths from 180 to 1100 nm. The lethal effect of pulsed light can be attributed to its rich broad-spectrum UVC content. This technology is also known as pulsed light, high intensity broad spectrum pulsed light, pulsed white light, pulsed UV light, and intense light pulses. IPL has been reported to inactivate *E. coli* O157:H7 in different fruits, vegetables, and drinks (Rajkovic et al. 2010).

5.6 Ultrasound

Ultrasound (US) treatments involve the application of sound waves of 20 kHz to foods in a liquid medium; these sound waves locally generate high pressures and temperatures, with the consequent lysis of microbial cells through intracellular cavitation. Destruction of *E. coli* O157:H7 cells has been studied in foods such as drinks and vegetables (Afari et al. 2016). In addition, Luna-Guevara et al. (2015) studied US efficacy to remove enterotoxigenic *E. coli* (ETEC) in tomatoes.

5.7 Radiation

One or more of three types of irradiation, namely, electron beam, X-ray, and gamma rays (Cobalt 60 and Cesium 137) have been approved for use with as many as 40 different foods to control microbial contamination in about 60 countries (Li et al. 2015). They are referred to as ionizing radiations. Microorganisms are inactivated primarily due to DNA damage, which destroys the reproductive capabilities and other functions of the cell. Different works have been conducted to analyze the effect of irradiation to control STEC O157:H7 and non-O157 in several foods (Trinetta et al. 2011).

5.8 Ozone

Probably, chemical agents are one of the most common interventions studied to minimize the transmission of STEC in foods. The FDA has approved the use of ozone (O3) as an antimicrobial agent for the treatment, storage, and processing of foods in gas and aqueous phases. It inactivates microorganisms through oxidization. The control of *E. coli* O157:H7 has been studied in drinks, vegetables, and fruits (Trinetta et al. 2011).

5.9 Organic Acids

The mechanism of inactivation by weak organic acids lays down in the ability of a nondissociated form of organic acid to penetrate through the cell membrane and to dissociate inside the cell, resulting in decreased intracellular pH value. Beside the decrease in intracellular pH, the perturbation of the membrane functions by organic acid molecules might be also responsible for microbial inactivation (Rajkovic et al. 2010). Organic acids can be applied as sprays, washes, or dipping solutions, depending on the food product to be decontaminated and on the infrastructure of the processing plant. Several studies have shown the effectiveness of different organic acids in the inactivation of *E. coli* O157:H7 in different foods (Mohan and Pohlman 2016; Fransisca and Feng 2012).

5.10 Other Emerging Processing Techniques

Among them, we can mention infrared heating to inactivate *E. coli* O157:H7 in ham (Ha et al. 2012); ohmic heating to control *E. coli* O157:H7 in juices (Park and Kang 2013); cold atmospheric plasma to inactivate *E. coli* O157:H7 in apples, cantaloupe, melons, and lettuce (Critzer et al. 2007); and electrolyzed oxidizing water and hot water to reduce *E. coli* O157:H7 and O26, O103, O111, and O145 in beef carcasses, meat and meat products (Kalchayanand et al. 2012), seeds and sprouts (Zhang et al. 2011), and strawberries and broccoli (Hung et al. 2010).

5.11 Hurdle Technology

The use of novel technologies alone is often insufficient to achieve adequate STEC inactivation in several foods. According to FDA regulations, novel technologies must accomplish at least a 5-log reduction of the pathogen of interest in order to be used as an alternative to pasteurization (FDA 2015). Sometimes, the use of two or more preservation factors applied simultaneously, known as hurdle technology, can

fulfill the requirements for a specific food product. Today, a number of novel technologies are good candidates for use in combination, and preliminary results have shown important effects on reducing STEC in foods. The use of multiple preservation techniques incorporating mild treatments can result in an enhanced preservative action by having an additive or synergistic effect on microbial inactivation (Tawema et al. 2016).

Food producers recognize that applying preharvest interventions with postharvest technologies for a "multihurdle" approach is the most effective way to minimize food contamination. Continued efforts in developing multiple hurdle or sequential intervention treatments will likely provide the greatest advances in minimizing the transmission of STEC through foods.

6 Regulation, Microbiological Criteria, and Methodologies to Detect and Isolate Diarrheagenic *E. coli*

The microbiological testing of finished food products alone is insufficient to guarantee their safety due to reasons concerned with sampling, methodology, and randomized distribution of microorganisms. Food safety must principally be ensured by a more preventative approach, such as product and process design and the application of the Good Hygiene Practices (GHP), the Good Manufacturing Practices (GMP), and the Hazard Analysis Critical Control Point (HACCP) principles (European Commission 2011).

Microbiological results cannot be expressed in absolute terms because samples are a portion of the batch of the food produced. Therefore, standards and standardized methodologies must be applied rigorously to guarantee them. Microbiology laboratories should use official methodology and adopt a quality management system. For example, ISO/IEC 17025:2005 specifies the general requirements for the competence to carry out tests and/or calibrations, including sampling, and is used to develop their management system for quality, administrative, and technical operations. It includes testing and calibration using standard, nonstandard, and laboratory-developed methods. Moreover, all methodologies and techniques used in food microbiology require validation, including conventional and rapid methods (AOAC International, BraCVAM, and Health Canada, among others). However, results should be interpreted according to microbiological criteria.

Microbiological criteria may be used to formulate design requirements and to indicate the required microbiological status of raw materials, ingredients, and end-products at any stage of the food chain as appropriate. These criteria should be applied by regulatory authorities and/or food business operators to distinguish between acceptable and unacceptable food. To establish a microbiological criterion, consideration should be given to the evidence of actual or potential hazards to health; the microbiological status of the raw material(s); the effect of processing on the microbiological status of the food; the likelihood and consequences of microbial

contamination and/or growth during subsequent handling, storage, and use; the category(s) of consumers concerned; the cost/benefit ratio associated with the application of the criterion; and the intended use of the food (FAO 1997).

The International Commission on Microbiological Specifications for Foods (ICMSF) has written extensively on the principles of controlling microbial hazards in foods (ICMSF 2011). These s-ame principles apply to the control of microorganisms associated with spoilage as well as general indicators of GHP/GMP. Some microbiological tests provide information regarding general contamination, incipient spoilage, or reduced shelf life. For instance, coliform counts have been widely used as universal indicators of hygiene, but in many products (e.g., meat or poultry, vegetables), *Enterobacteriaceae* will inevitably be present and the apparently high coliform counts do not necessarily indicate hygienic failure or consumer risk (ICMSF 2011).

E. coli is used to indicate recent fecal contamination or unsanitary processing. However, the presence of a low content of generic *E. coli* is inevitable in several products, and this is the reason for a limit of tolerance when counting this group of bacteria in some foods, such as meat or poultry. Generic *E. coli* is considered a low risk microorganism group and identified as an indirect hazard (ICMSF 2011).

Sampling plans become increasingly more stringent with increased severity. The following terms are used: n = the number of sample units to be analyzed; c = the maximum number of sample units allowable with marginal but acceptable results (i.e., between m and M); m = concentration separating good quality or safety from marginally acceptable quality; M = concentration separating marginally acceptable quality from unacceptable quality or safety.

The ICMSF proposes the following sampling plan for generic *E. coli*: (a) risk is reduced ($n = 5$, $c = 3$), (b) no change in risk ($n = 5$, $c = 2$), and may increase risk ($n = 5$, $c = 1$). Several counting methods for generic *E. coli* (Feng et al. 2013) and different microbiological criteria according to food products are used. For example, the *E. coli* test results for a chicken slaughter establishment will be acceptable if they are not above 100 cfu/mL, marginal if above 100 cfu/mL but not above 1000 cfu/mL, and unacceptable if above 1000 cfu/mL (FSIS 2014). The European Commission (EC) Regulation No 2073/2005 on microbiological criteria for food products establishes the criteria for *E. coli* in several foodstuffs, namely, minced meat ($n = 5$, $c = 2$, $m = 50$ cfu/g, $M = 500$ cfu/g) and meat preparations ($n = 5$, $c = 2$, $m = 500$ cfu/g, $M = 5000$ cfu/g). *E. coli* O157:H7 is the DEC most often implicated in illnesses worldwide (Majowicz et al. 2014); since the infectious dose estimated for this serotype is 10–100 cells (Feng et al. 2015), the ICMSF considered O157:H7 as a severe hazard for consumers, proposing the following sampling plan according to food handling and consumption conditions: (a) reduced risk ($n = 15$, $c = 0$), (b) no change in risk ($n = 30$, $c = 0$), and may increase risk ($n = 60$, $c = 0$) (ICMSF 2011).

Microbiological criteria must be updated constantly considering the emergence of pathogens that affect the health of consumers. For example, the first *E. coli* O157:H7 outbreak in 1993 in the US was associated with undercooked hamburgers from a fast food chain; it prompted the Food Safety and Inspection Service (FSIS

1996) to declare this microorganism as an adulterant in ground beef and initiated a screening program at raw ground meat processing plant level and at retail outlets. In order to lower the incidence of *E. coli* O157:H7 disease, the FSIS increased the amount of ground beef samples analyzed from 25 to 325 g ($n=5$, $c=0$), making the laboratory methodology more stringent, and introduced a more sensitive laboratory test for recovery of *E. coli* O157:H7 based on immunomagnetic separation. The detection of this bacterium improved and a low amount of *E. coli* O157:H7 could be isolated. The number of recalls dropped and remained controlled until 2002. Then, HACCP plans and other mitigation strategies taken by FSIS to reduce the prevalence of *E. coli* O157:H7 were reassessed, such as sanitary procedures of dressing and interventions with antimicrobials (USDA 2013). Presently, the FSIS uses the most rigorous sampling plan for raw ground beef and trimmings ($n=60$, $c=0$) (FSIS 2014). In addition, the FDA performs *E. coli* O157:H7 search and monitoring in different food matrices in the US (http://www.fda.gov/Food). Due to food trade requirements, particularly meat, many countries of America, such as Argentina, Brazil, Canada, Chile, Mexico, Colombia, Costa Rica, Nicaragua, and Uruguay also perform those procedures (USDA 2016). These countries have the analytical capacity to detect, isolate, and characterize *E. coli* O157:H7. In Argentina, the Argentine Food Code (AFC) not only complies with international trade requirements, but also contains several microbiological criteria that include the absence of *E. coli* O157:H7 in several food products (http://www.anmat.gov.ar/alimentos/normativas_alimentos_caa.asp). The microbiological methodologies recommended for the detection and isolation of *E. coli* O157:H7 in the AFC are USDA MLG 5.09, BAM-FDA 2011, and ISO 16654:2001.

Due to the increase of cases and outbreaks associated with the consumption of food products contaminated with STEC non-O157, food safety agencies focus their efforts in the search of strains most frequently identified in clinical infections from food. At present, several official protocols are used to find STEC in meat products, including only some serogroups, such as O26, O45, O103, O104, O111, O121, O145, and O157 (USDA MLG 5B.05:2014, BAM-FDA 2011, ISO/TS 13136:2012). Consequently, commercial and noncommercial RT-PCR tests are based on the detection of *stx* and *eae* genes and the STEC serogroups (Auvray et al. 2009; Brusa et al. 2015).

The USDA developed validated protocols for the detection, isolation, and characterization of STEC O26, O45, O103, O111, O121, and O145 from ground beef and trimmings (USDA MLG 5B.05). In addition, the FDA proposed a standard for DEC detection and isolation from foods, which is the only one together with EHEC that considers ETEC, EPEC, and EIEC strains. The Bacteriological Analytical Manual describes a methodology for O157:H7 isolation and identification, and a different one for the rest of DEC, including STEC non-O157 strains (BAM-FDA 2011). In the past, the FDA regulatory position focused only on O157:H7; however, the presence of any pathogenic STEC in products regulated by the agency is of concern, so it is essential to isolate, do additional testing and discern EHEC from STEC strains that have not been implicated in illnesses and may not be pathogenic.

The same as with *E. coli* O157:H7, sanitary control agencies of the countries that sell trimming and/or raw ground beef to the USA and other destinations, officially implemented the analysis of STEC non-O157 according to the country of destination. Many countries of America have the analytical capacity to detect, isolate, and characterize STEC non-O157. In Argentina, the National Commission on Foods recently approved new microbiological criteria in the AFC for food products consumed in Argentina, specifying the absence of STEC non-O157 as a target for the prevention of prevalent serogroups in the country. These microbiological criteria were established for raw ground beef, RTE food, sausages, and minimally processed fruits and vegetables. The serogroups included in the proposed AFC modification are O145, O121, O26, O111, and O103, whereas the microbiological methodologies recommended in AFC are USDA MLG 5B.05, BAM-FDA 2011, and ISO/TS 13136: 2012.

All official methodologies to find *E. coli* O157:H7 and STEC non-O157 in food products contain the following general steps: (1) enrichment in selective broth, (2) screening, (3) immunomagnetic separation (IMS), (4) isolation in selective and differential agar, (5) confirmation by biochemical and serological tests, and (6) identification of virulence factors. A summary with all these official methodologies is presented in Table 8.2.

During food processing, the technological processes applied, such as freezing, can cause injury and stress in bacterial cells. The enrichment step is necessary to stimulate the growth of potentially stressed STEC cells by increasing their number, and further dilute the effects of inhibitors and competitive background bacteria that may be present in the sample (Wang et al. 2013). The selective agents and inhibitors must be in adequate quantities to curb the proliferation of competitive background bacteria, allowing the growth of stressed STEC cells. In addition, STEC heterogeneity determines that selective enrichment broths for some serogroups can inhibit the development of others (Feng et al. 2015). A single genetic marker that can separate pathogenic and nonpathogenic *E. coli* strains has not yet been identified. However, most STEC strains frequently associated with severe disease in humans correspond to specific somatic antigens (O-Ag) and possess *stx* and *eae* genes. These genotypic virulence characteristics have been used to develop screening methods based on molecular detection strategies (Andreoletti et al. 2013). Negative samples by the screening test can be reported as negative. Updated information about the virulence profile of strains isolated from cases and outbreaks is necessary to identify other virulence markers that should be searched for in food through the screening test. Although many unofficial PCR assays have been developed, reference methods are subject to extensive validation processes to ensure their results. Rapid STEC screening and isolation methods in food samples have been developed as a result of the public health impact of some STEC serogroups and the available standards for their search in food products, namely, immunoassay-based method (lateral flow), molecular biology-based method (*stx*, *eae*, and specific Ag-O gene detection by PCR), and immuno- and molecular biology-based method (IMS and PCR). The simultaneous detection of virulence genes in one food sample is not enough to determine that a food is contaminated with pathogenic STEC. These

Table 8.2 Official methodologies and criteria to analyze diarrheagenic *E. coli* in food

	Bacteria	Enrichment	Screening	Criteria	Isolation	Characterization	Food product
USDA							
MLG 5.09	STEC:	mTSB 42 ± 1 °C, 15–24 h	– O157:H7 RT-PCR	Samples (+) for O157:H7: isolation	IMS + mRBA	– Latex agglutination assays	– Raw beef, raw beef mixes, beef trim, and trim components, raw ground beef and raw pork or poultry mixes
	O157:H7		– Lateral Flow *E. coli* O157: (including H7)		Optional: post-IMS acid treatment step to reduce background flora	– Biochemical confirmation – Shiga toxin/toxin genes confirmation	– Environmental sponges and carcass sponges
MLG 5B.05	STEC:		*stx* and *eae* RT-PCR	Samples (+) or (–) for *stx* and *eae*, and (–) for all serogroup: (–) sample			
	O26		Samples (–): reported as (–)	Samples (+) for *stx* and *eae*, and for at least one serogroup: isolation			
	O45		Samples (+): analyzed by RT-PCR for serogroup				
	O103						
	O111						
	O121						
	O145						
FDA-BAM Chapter 4A:2011	EHEC	mBPWp 37 °C ± 1 °C, 5 h	RT-PCR: stx_1, stx_2 and the +93 SNP in the *uidA* gene	"Probable (+) O157": +93 *uidA* SNP (+) by itself or with stx_1 and/or stx_2	"Probable (+) O157": IMS + TC-SMAC and a chromogenic agar	Typical colonies, X-gal (+), MUG (–) and indole (+): antisera for O157 and H7 antigens	Food

(continued)

Table 8.2 (continued)

	Bacteria	Enrichment	Screening	Criteria	Isolation	Characterization	Food product
	STEC	Added ACV supplement 42 °C ± 1 °C, overnight		"Probable (+) STEC": +93 *uidA* SNP (−) and but stx_1 and/or stx_2 (+)	"Probable (+) STEC": L-EMB agar and a chromogenic agar	Typical or hemolytic colonies and X-gal (+), MUG (+) or (−) and indole (+): additional testing for virulence factors and for major serogroups of concern	
				In both cases continue with isolation	Additional agar: SHIBAM	PFGE	
						In all cases: biochemical confirmation and verify toxigenic potential	
	ETEC	BHI 35 °C, 3 h	–	–	L-EMB and MAC agars	EIEC: confirm invasive potential of the isolates[a]	
	EPEC	Double strength TPB 44.0 ± 0.2 °C, 20 h			Biochemical and morphological identification of *E. coli*	EPEC: A/E and localized adherence[b] and absence of Stx[c]	
	EIEC						
ISO							
16654:2001	STEC:	mTSB + 20	–	–	IMS + TC-SMAC and the user's choice of the second selective isolation agar	Indole production and agglutination with *E. coli* O157 antiserum	Food
	O157	41.5 °C ± 1 °C, 18–24 h				Isolates may be sent to reference laboratory for further characterization	

13136:2012	STEC:	mTSB + 16	Step 1: RT-PCR for *stx* and *eae*	Samples *stx* (−): procedure stopped	IMS[d] + TBX agar	Confirm the presence of *eae* and serogroup if identified in the screening step
	O26	BPW or mTSB + 12A	Step 2: RT-PCR for molecular serogrouping	If isolation is NOT achieved from (+) samples:	Optional:	Isolates may be sent to reference laboratory for further characterization
	O103	37 ± 1 °C, 18–24 h		– Samples *stx* (+): presumptive detection of STEC	STEC O26: RMAC	
	O111			– Samples *stx* and *eae* (+): presumptive detection of STEC	All: EHA, detecting enterohemolysin production	
	O145			– Samples *stx, eae* and gene associated to serogroup (+): presumptive detection of STEC of XX serogroup		
	O157					

+ positive, − negative, *mTSB* modified tryptone soya broth, *IMS* immunomagnetic separation, *mRB* modified Rainbow agar, *BPW* buffered peptone water, *mBPWp* modified BPW with pyruvate, *ACV* acriflavin-cefsulodin-vancomycin, *SNP* single nucleotide polymorphism, *MAC* MacConkey agar, *TC-SMAC* MAC with sorbitol, cefixime and tellurite, *L-EMB* levine eosin methylene blue agar, *SHIBAM* STEC heart infusion washed blood agar with Mitomycin-C, *PFGE* pulsed field gel electrophoresis, *BHI* broth heart infusion, *TPB* tryptone phosphate broth, *mTSB + 20* mTSB supplemented with 20 mg/L of novobiocin plus casamino acids, *mTSB + 16* mTSB supplemented with 16 mg/L of novobiocin plus casamino acids, *mTSB + 12A* mTSB supplemented with 12 mg/L of acriflavin, *TBX* tryptone bile glucuronic agar, *RMAC* differential solid medium containing rhamnose instead of lactose, *EHA* enterohemolysin agar

[a]By Sereny test or the Guinea pig keratoconjunctivitis assay (Nataro and Kaper 1998), HeLa tissue culture cell assay (Mehlman et al. 1982), or with the in vitro staining technique using acridine orange to stain intracellular bacteria in HeLa monolayers (Miliotis and Feng 1993)

[b]Using Hep-2 or HeLa tissue cells

[c]Using tests outlined for EHEC

[d]In case of positive to genes associated with the serogroups in the scope of the method

virulence factors could potentially come from two or more bacterial strains; therefore, isolation is necessary to confirm a positive result. IMS, a rapid isolation method that uses magnetic particles coated with specific antibodies against STEC O-Ag (O26, O45, O103, O104, O111, O121, O145, and O157), is combined with chromogenic agars to improve STEC isolation.

Due to the emergence of strains carrying the *stx* genes and associated with severe disease, such as *E. coli* O104:H4, ISO/TS 13136:2012 considers that all *stx*-positive strains should be considered pathogenic (Frank et al. 2011) and proposes to continue with isolation. However, the mere detection of *stx*-genes as a definitive or confirmatory test for STEC presence might be excessive and STEC could be overdiagnosed. For example, numerous reports about STEC and meat demonstrate poor correlation between the number of positive samples during *stx* screening and the number of samples that can be confirmed by isolation (Bosilevac et al. 2007; Bosilevac and Koohmaraie 2011; Pradel et al. 2000). Successful STEC confirmation by culture isolation in ground beef was 8/25 (32.0 %) in Argentina (Brusa et al. 2016), 300/1006 (29.8 %) in the US (Bosilevac and Koohmaraie 2011), and 16/47 (34.0 %) in France (Pradel et al. 2000); in boneless beef trim used for ground beef, it was 41/237 (17.2 %) in the US, Australia, and New Zealand, and 40/72 (55.5 %) in Uruguay (Bosilevac et al. 2007). Factors such as low detection limit of the screening technique and high levels of background bacteria could impair the accurate detection of STEC in contaminated meat samples (Auvray et al. 2009).

Isolation might not be ideal, and that there is no single method or combination of isolation methods capable of identifying all STEC serogroups. Therefore, in order to maximize the use of the available detection and isolation tools, it would be necessary not only to combine multiple bacteriological tools but also adapt the most adequate set of techniques based on the regional prevalence of specific STEC that affect human health. New technologies combining molecular approaches that allow the identification of numerous virulence genes, rapid microbiological methods and traditional bacteriology should be considered to improve isolation.

7 Control of Disease Determinants

Foodborne diseases are common in all American countries, but the mere exposure to a pathogen does not necessarily mean that a person will get sick. There are many variables related with the host, the agent and the environment that produce a change in the severity or frequency of the disease. Identifying these factors allow manipulation to maximize health or prevent diseases.

Molecular epidemiology and microbial genomics have facilitated the characterization and comparison of strains, such as *E. coli* O157:H7 strains isolated from human and animal sources. Different studies identify the *E. coli* O157:H7 factors that influence successful human infection. Characterization of Shiga toxin genotypes, locus-specific polymorphism assays, allelic variation of virulence genes, clade typing, and Shiga toxin bacteriophage insertion site analysis have been used

to characterize bacterial properties (Mellor et al. 2012). *E. coli* O157:H7 strains carrying Shiga toxin stx_2 gene predominate in human infection, being responsible for more severe disease symptoms than stx_{2c} strains. However, Manning et al. (2008) identified a hyper-virulent clade of *E. coli* O157:H7 isolated from US outbreaks associated with raw spinach consumption. Other studies conducted in Argentina have demonstrated that *E. coli* O157:H7 strains belonging to clade 8 are predominant in both Argentinean cattle and human cases, where such strains are implicated in >90 % of postenteric HUS (Mellor et al. 2012). In another study conducted in Argentina, clade 8 *E. coli* O157:H7 strains from butcher shops were predominantly isolated, but HUS epidemiological data in the same geographical area did not reveal the presence of severe cases of disease in humans (Galli L, personal communication 2016).

The available molecular data about STEC strains in the reservoir (cattle) and in clinical cases provide invaluable information that can be used to monitor the circulating strains and to evaluate genetic changes and variations in the presence of virulence determinants, which is helpful to analyze the spread of the pathogen through different agro-food chains.

A number of risk factors associated with DEC infections have also been identified by epidemiological studies, providing the scientific basis to recommend measures for the prevention of possible human exposure to foodborne STEC infection. Thorough cooking of meat, particularly ground meat (Voetsch et al. 2007), avoidance of cross-contamination during food preparation (Signorini and Fizzo 2009), access to safe drinking water, consumption of a varied diet that includes fruits and vegetables (Andreoletti et al. 2013), and public education on the importance of good personal hygiene and cleanliness of eating utensils (Rivas et al. 2011) are among the measures that complement the actions aimed at lowering the prevalence of STEC in cattle by observing good hygiene practices during slaughter in meat processing plants and by careful preparation of meat for distribution to consumers (Bentancor et al. 2012).

Regarding the importance of public education programs, an interesting pilot program called “Healthy Butcher Shop” was implemented in a city of the province of Buenos Aires, Argentina (Ortega et al. 2014) to evaluate butcheries integrally. The program included risk quantification and determination of the bacteriological quality of raw ground beef and environmental samples, implementation of specific improvement actions in butcheries and consumer information, and verification of the impact of improvement actions. The program successfully improved the sanitary conditions of butcher shops, reducing the count of the principal indicator microorganisms (mesophilic aerobic organisms, *E. coli* and *Staphylococcus aureus* positive coagulase), and the presence of pathogens (*Salmonella* spp., *E. coli* O157:H7, STEC non-O157, and *L. monocytogenes*) in ground meat and the environment (meat tables, knives, meat mincing machines, and manipulator hands).

Additionally, a study conducted to compare the presentation and distribution of factors hypothetically associated with HUS in patients living in urban and rural areas of Argentina identified some interesting risk factors. HUS incidence rate was significantly higher in the rural population and median age was significantly lower

in children from the rural area. This could be explained by a more frequent contact with bovine feces, raw milk consumption, and a higher proportion of relatives who work in risk labors in rural populations (Rivero et al. 2013).

8 Quantitative Risk Assessment

Risk analysis is a valuable tool in the management of microbial food safety problems and can provide a systematic approach for regulatory authorities and the food industry to control the risk associated to a pathogen in a particular food commodity (Duffy et al. 2006). Risk analysis tools have proven effectiveness to reduce foodborne diseases through the design, development, implementation, evaluation, and communication of control measures to protect the public health of a country or region (Cherry et al. 2014).

In America, four quantitative risk assessments for STEC in ground beef were designed. Cassin et al. (1998) and Smith et al. (2013) developed a model for the production of ground beef considering the Canadian conditions. Ebel et al. (2004) conducted a quantitative risk assessment for *E. coli* O157:H7 in ground beef in the US. Finally, Signorini and Tarabla (2009) performed a quantitative risk assessment for STEC in ground beef considering the particular situation in Argentina. All models reported a similar probability of illness from consumption of a single serving of beef contaminated with STEC.

However, the probability of infection is not by itself relevant and useful information. An additional evaluation (sensibility analysis) can be performed during quantitative risk assessment to identify input variables of the model with bigger impact on the output (probability of infection) and thus determine the stages of the agrofood chain on which management measures should be focused (Luna-Carrasco et al. 2009). The models demonstrated that the factor having the largest impact on predicted illness was the concentration of pathogen in the feces or hide of cattle in slaughterhouses.

Cooking preference was identified as a highly sensitive factor (Cassin et al. 1998). However, Signorini and Tarabla (2009) observed that this factor had a negligible influence on the outcome, based on the facts that Argentinean consumers prefer a well-done steak, and almost 40 % of Canadian consumers prefer hamburgers cooked rare to medium rare. While epidemiological evidence indicates that ground beef is the most important foodborne exposure source to STEC, some studies suggest that these organisms may also survive under customary cooking practices. In contrast, the meat interior of intact products remains essentially protected from pathogens and, consequently, customary cooking of the intact product will destroy any *E. coli* (FSIS 2002). Smith et al. (2013) estimated that the average probability of illness per serving of ground beef was 263 and 2965 times greater than that associated with nonintact (tenderized) and intact beef cuts, respectively. Additionally, consumption of a serving of nonintact beef cuts was associated with a greater average risk (approximately 11 times) than consumption of intact beef cuts.

Finally, quantitative risk models are helpful to assess the effect of applying a new hypothetical risk management measure at a specific point in the agro-food chain to establish probability of DEC infection (Duffy et al. 2006; Luna-Carrasco et al. 2009). This tool can be used to evaluate an essentially limitless combination of intervention scenarios.

Preslaughter interventions (e.g., vaccination, use of agents that suppress or reduce the shedding of STEC in feces) would have the greatest potential impact to reduce the entry of STEC and hence the risk of STEC infection in consumers (Signorini and Tarabla 2010). The most effective management measure to reduce the risk of STEC infection was the reduction of pathogen load in the cattle hide. Management measures to control the pathogen once present in the meat are inefficient to reduce the risk of zoonosis. The intensification of livestock production could result in greater public health risk, given the increased exposure to the pathogen generated by these practices (Signorini and Tarabla 2010). On the other hand, Koohmaraie et al. (2007) preferred postharvest to preharvest intervention, specifically due to the universality of postharvest intervention as opposed to the specificity of preharvest interventions.

Currently, STEC risk assessment is limited by lack of data in critical areas. All models need more data about the pathogen in the agro-food chain, epidemiological data on outbreaks and sporadic cases, and dose–response models which more accurately describe the disease in humans and risk groups (Duffy et al. 2006). Quantitative risk assessment models can provide risk managers and policy-makers with a comprehensive picture of the key factors in the agro-food chains of interest (Smith et al. 2013).

A multidisciplinary approach is required to generate evidence-based risk management measures and thus achieve a strategic control of DEC infections. In this way, it will be possible to protect the health of consumers. It is important to recognize that apart from beef, other meats, food, water, and indirect sources (animal contact, recreational, person to person) also play a role in the transmission of this pathogen, and they also need to be addressed to reduce its impact on public health (Duffy et al. 2006).

Public health epidemiological surveillance establishes a relationship between the illness and its source. Strengthening epidemiological surveillance based on molecular analysis would help obtain more accurate information about virulence genes and their clinical implications, as well as DEC prevalence in food products. It is essential to conduct periodic reviews of the new epidemiological information and verify that food analyses are appropriate to identify strains affecting the population. It would be necessary to not only combine new molecular approaches, rapid microbiological methods, and traditional bacteriological tools, but also adapt the most adequate set of techniques based on the regional prevalence of specific DEC strains that affect human health in order to maximize the use of the available tools for their detection and isolation from food products.

Acknowledgments The authors acknowledge A. Di Maggio IGEVET (UNLP-CONICET LA PLATA), Facultad de Ciencias Veterinarias UNLP for manuscript correction.

References

Aertsen A, Michiels CW (2004) Stress and how bacteria cope with death and survival. Crit Rev Microbiol 30:263–273

Afari GK, Hung YC, King CH, Hu A (2016) Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT 104 on fresh produce using an automated washer with near neutral electrolyzed water and ultrasound. Food Control 63:246–254

Ait-Ouazzou A, Espina L, García-Gonzalo D, Pagán R (2013) Synergistic combination of physical treatments and carvacrol for *Escherichia coli* O157:H7 inactivation in apple, mango, orange, and tomato juices. Food Control 32:159–167

Alonso MZ, Lucchesi P, Rodríguez E, Parma AE, Padola NL (2012) Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli* (STEC) in broiler chickens and derived products at different retail stores. Food Control 23:351–355

Amézquita-Montes Z, Tamborski M, Kopsombut UG, Zhang C, Arzuza OS, Gómez-Duarte OG (2015) Genetic relatedness among *Escherichia coli* pathotypes isolated from food products for human consumption in Cartagena, Colombia. Foodborne Pathog Dis 12:454–461

Ammon A, Makela P (2010) Integrated data collection on zoonoses in the European Union, from animals to humans, and the analyses of the data. Int J Food Microbiol 139:43–47

Andreoletti O, Baggesen DL, Bolton D, Butaye P, Cook P, Davies R, Fernandez-Escamez PS, Griffin J, Hald T, Havelaar A, Koutsoumanis K, Lindqvist R, McLauchlin J, Nesbakken T, Prieto-Maradona M, Ricci A, Ru G, Sanaa M, Simmons M, Sofos J, Threlfall J (2013) Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA J 11:3138 (106p)

Auvray F, Lecureuil C, Dilasser F, Taché J, Derzelle S (2009) Development of a real-time PCR assay with an internal amplification control for the screening of Shiga toxin-producing *Escherichia coli* in foods. Lett Appl Microbiol 48:554–559

Barbosa LJ, Ribeiro LF, Lavezzo LF, Barbosa MM, Rossi GA, do Amaral LA (2016) Detection of pathogenic *Escherichia coli* and microbiological quality of chilled shrimp sold in street markets. Lett Appl Microbiol 62:372–378

Bentancor AB, Ameal LA, Calviño MF, Martínez MC, Miccio L, Degregorio OJ (2012) Risk factors for Shiga toxin-producing *Escherichia coli* infections in preadolescent schoolchildren in Buenos Aires, Argentina. J Infect Dev Ctries 6:378–386

Boletín Integrado de Epidemiología N° 298 (2016) Vigilancia integrada de Síndrome Urémico Hemolítico (SUH). Ministerio de Salud de Argentina. http://www.msal.gob.ar/images/stories/boletines/Boletin-Integrado-De-Vigilancia-N298-SE8.pdf

Bosilevac JM, Koohmaraie M (2011) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. Appl Environ Microbiol 77:2103–2112

Bosilevac JM, Guerini MN, Brichta-Harhay DM, Arthur TM, Koohmaraie M (2007) Microbiological characterization of imported and domestic boneless beef trim used for ground beef. J Food Prot 70:440–449

Briñez WJ, Roig-Sagués AX, Herrero MMH, Lopez BG (2006) Inactivation by ultrahigh-pressure homogenization of *Escherichia coli* strains inoculated into orange juice. J Food Prot 69:984–989

Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. J Infect Dis 192:1422–1429

Brusa V, Aliverti V, Aliverti F, Ortega EE, de la Torre JH, Linares LH, Etcheverría AI, Padola NL, Galli L, Peral-García P, Leotta GA (2013) Shiga toxin-producing *Escherichia coli* in beef retail markets from Argentina. Front Cell Infect Microbiol 2:171

Brusa V, Galli L, Linares LH, Ortega EE, Lirón JP, Leotta GA (2015) Development and validation of two SYBR green PCR assays and a multiplex real-time PCR for the detection of Shiga toxin-producing *Escherichia coli* in meat. J Microbiol Methods 119:10–17

Brusa V, Piñeyro PE, Galli L, Linares LH, Ortega EE, Padola NL, Leotta GA (2016) Isolation of Shiga toxin-producing *Escherichia coli* from ground beef using multiple combinations of enrichment broths and selective agars. Foodborne Pathog Dis 13:163–170

Canizalez-Roman A, Gonzalez-Nuñez E, Vidal JE, Flores-Villaseñor H, León-Sicairos N (2013) Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern Mexico. Int J Food Microbiol 164:36–45

Cassin MH, Lammerding AM, Todd ECD, Ross W, McColl RS (1998) Quantitative risk assessment for *Escherichia coli* O157:H7 in ground beef hamburgers. Int J Food Microbiol 41:21–44

Castro-Rosas J, Cerna-Cortés JF, Méndez-Reyes E, Lopez-Hernandez D, Gómez-Aldapa CA, Estrada-Garcia T (2012) Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. Int J Food Microbiol 156:176–180

Cherry C, Hofelich-Mohr A, Lindsay T, Diez-González F, Hueston W, Sampedro F (2014) Knowledge and perceived implementation of food safety risk analysis framework in Latin America and the Caribbean region. J Food Prot 77:2098–2105

Comery R, Thanabalasuriar A, Garneau P, Portt A, Boerlin P, Reid-Smith RJ, Harel J, Manges AR, Gruenheid S (2013) Identification of potentially diarrheagenic atypical enteropathogenic *Escherichia coli* strains present in Canadian food animals at slaughter and in retail meats. Appl Environ Microbiol 79:3892–3896

Critzer FJ, Kelly-Wintenberg K, South SL, Golden DA (2007) Atmospheric plasma inactivation of foodborne pathogens on fresh produce surfaces. J Food Prot 70:2290–2296

Duffy G, Cummins E, Nally P, O'Brien S, Butler F (2006) A review of quantitative microbial risk assessment in the management of *Escherichia coli* O157:H7 on beef. Meat Sci 74:76–88

Ebel E, Schlosser W, Kause J, Orloski K, Roberts T, Narrod C, Malcolm S, Coleman M, Powell M (2004) Draft risk assessment of the public health impact of *Escherichia coli* O157:H7 in ground beef. J Food Prot 67:1991–1999

European Commission—Food and Feed Safety (2011) Microbiological criteria. http://ec.europa.eu/food/food/biosafety/salmonella/microbio_en.htm

FAO (1997) Principles for the establishment and application of microbiological criteria for foods. http://www.fao.org/docrep/w6419e/w6419e04.htm

FAO (2001) The state of food insecurity in the world. http://www.fao.org/docrep/003/y1500e/y1500e00.htm

FAO/WHO (2003) Assuring food safety and quality: guidelines for strengthening national food control systems. http://www.fao.org/docrep/006/y8705e/y8705e00.htm

FDA (2015) Hazard analysis and critical control point (HACCP) systems. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=120.24

Feng PC, Reddy SP (2014) Prevalence and diversity of enterotoxigenic *Escherichia coli* strains in fresh produce. J Food Prot 77:820–823

Feng P, Weagant SD, Grant MA, Burkhardt W (2013). Bacteriological analytical manual, Chapter 4, Enumeration of *Escherichia coli* and the Coliform Bacteria. U.S. Food and Drug Administration. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm

Feng P, Weagant SD, Jinneman K (2015). Bacteriological analytical manual, Chapter 4A, Diarrheagenic *Escherichia coli*. U.S. Food and Drug Administration. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm

Frank C, Faber MS, Askar M, Bernard H, Fruth A, Gilsdorf A, Hohle M, Karch H, Krause G, Prager R, Spode A, Stark K, Werber D (2011) Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011. Euro surveill 16(21). pii:19878

Fransisca L, Feng H (2012) Effect of surface roughness on inactivation of *Escherichia coli* O157:H7 87-23 by new organic acid-surfactant combinations on alfalfa, broccoli, and radish seeds. J Food Prot 75:261–269

FSIS (1996) Guidelines for *Escherichia coli* testing for process control. Verification in poultry slaughter establishments. http://www.fsis.usda.gov/wps/wcm/connect/3efc7f8e-e6a2-4997-91ba-9c579c2a1f14/Guideline_for_Ecoli_Testing_Slaughter_Estab.pdf?MOD=AJPERES

FSIS (2002) Comparative risk assessment for intact (non-tenderized) and non-intact (tenderized) beef. http://www.fsis.usda.gov/shared/PDF/Beef_Risk_Assess_Report_Mar2002.pdf

FSIS (2014) FSIS compliance guideline for establishments sampling beef trimmings for Shiga toxin-producing *Escherichia coli* (STEC) organisms or virulence markers. http://www.fsis.usda.gov/wps/wcm/connect/e0f06d97-9026-4e1e-a0c2-1ac60b836fa6/Compliance-Guide-Est-Sampling-STEC.pdf?MOD=AJPERES

Gaulin C, Levac E, Ramsay D, Dion R, Ismaïl J, Gingras S, Lacroix C (2012) *Escherichia coli* O157:H7 outbreak linked to raw milk cheese in Quebec, Canada: use of exact probability calculation and case-case study approaches to foodborne outbreak investigation. J Food Prot 75:812–818

Gil AI, Lanata CF, Hartinger SM, Mäusezahl D, Padilla B, Ochoa TJ, Lozada M, Pineda I, Verastegui H (2014) Fecal contamination of food, water, hands, and kitchen utensils at the household level in rural areas of Peru. J Environ Health 76:102–106

Gómez-Aldapa CA, Torres-Vitela M, Acevedo-Sandoval OA, Rangel-Vargas E, Villarruel-López A, Castro-Rosas A (2013) Presence of Shiga toxin–producing *Escherichia coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, and enterotoxigenic *E. coli* on tomatoes from public markets in Mexico. J Food Prot 76:1621–1625

Gómez-Aldapa CA, Cerna-Cortes JF, Rangel-Vargas E, Torres-Vitela MR, Villarruel-López A, Gutiérrez-Alcántara EJ, Castro-Rosas J (2016) Presence of multidrug-resistant Shiga toxin-producing *Escherichia coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli*, on raw nopalitos (*Opuntia ficus-indica* L.) and in nopalitos salads from local retail markets in Mexico. Foodborne Pathog Dis 13:269–274

Gómez-Aldapa CA, Rangel-Vargas E, León BD, Castro-Rosas J (2014) Presence of non-O157 Shiga toxin-producing *Escherichia coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli* and *Salmonella* in fresh beetroot (*Beta vulgaris* L.) juice from public markets in Mexico. J Sci Food Agric 94:2705–2711

Ha JW, Ryu SR, Kang DH (2012) Evaluation of near-infrared pasteurization in controlling *Escherichia coli* O157:H7, *Salmonella enterica* Serovar Typhimurium, and *Listeria monocytogenes* in ready-to-eat sliced ham. Appl Environ Microbiol 78:6458–6465

Heiman KE, Mody RK, Johnson SD, Griffin PM, Gould LH (2015) *Escherichia coli* O157 outbreaks in the United States, 2003-2012. Emerg Infect Dis 21:1293–1301

Hung YC, Tilly P, Kim C (2010) Efficacy of electrolyzed oxidizing (EO) water and chlorinated water for inactivation of *Escherichia coli* O157:H7 on strawberries and broccoli. J Food Qual 33:559–577

ICMSF (2011) Microorganisms in foods 8: use of data for assessing process control and product acceptance. Springer, New York

IFT (2002) Expert report on emerging microbiological food safety issues. Implications for control in the 21st century. IFT Expert Report. www.ift.org

ISO 16654:2001. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Escherichia coli* O157

ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories

ISO/TS 13136:2012. Microbiology of food and animal feed. Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens. Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups

Kalchayanand N, Arthur T, Bosilevac J, Schmidt J, Wang R, Shackelford S, Wheeler T (2012) Evaluation of commonly used antimicrobial interventions for fresh beef inoculated with Shiga toxin-producing *Escherichia coli* serotypes O26, O45, O103, O111, O121, O145, and O157:H7. J Food Prot 75:1207–1212

Koohmaraie M, Arthur TM, Bosilevac JM, Brichta-Harhay DM, Kalchayanand N, Shackelford SD, Wheeler TL (2007) Interventions to reduce/eliminate *Escherichia coli* O157:H7 in ground beef. Meat Sci 77:90–96

Li S, Kundu D, Holley RA (2015) Use of lactic acid with electron beam irradiation for control of *Escherichia coli* O157:H7, non-O157 VTEC *E. coli*, and *Salmonella* serovars on fresh and frozen beef. Food Microbiol 46:34–39

Line J, Fain A, Moran A, Martin L, Lechowich L, Carosella J, Brown W (1991) Lethality of heat to *Escherichia coli* O157:H7: D-value and z-value determinations in ground beef. J Food Prot 54:762–766

Luna-Carrasco J, Signorini-Porchietto M, Díaz-García R, Ordoñez-Méndez LB (2009) Risk assessment in food. ILSI México, México, p 43

Luna-Guevara ML, Luna-Guevara JL, Ruiz-Espinosa H, Leyva-Abascal L, Díaz-González CB (2015) Efficacy of disinfection treatments using essential oils and ultrasound on tomato fruits inoculated with *Escherichia coli* and impact on antioxidant activity. Rev Argent Microbiol 47:251–255

Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD (2014) Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. Foodborne Pathog Dis 11:447–455

Manning SD, Motiwala AS, Springman AC, Qi W, Lacher DW, Ouelette LM, Mladonicky JM, Somsel P, Rudrik JT, Dietrich SE, Zhang W, Swaminathan B, Alland D, Whittam TS (2008) Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc Natl Acad Sci U S A 105:4868–4873

Mehlman IJ, Romero A, Atkinson JC, Aulisio C, Sanders AC, Campbell W, Cholenski J, Ferreira J, Forney E, O'Brian K, Palmieri M, Weagant S (1982) Detection of invasiveness of mammalian cells by *Escherichia coli*: collaborative study. J Assoc Off Anal Chem 65:602–607

Mellor GE, Sim EM, Barlow RS, D'Astek BA, Galli L, Chinen I, Rivas M, Gobius KS (2012) Phylogenetically related Argentinean and Australian *Escherichia coli* O157 isolates are distinguished by virulence clades and alternative Shiga toxin 1 and 2 prophages. Appl Environ Microbiol 78:4724–4731

Miliotis MD, Feng P (1993) In vitro staining technique for determining invasiveness in foodborne pathogens. FDA LIB 3754

Mohan A, Pohlman FW (2016) Role of organic acids and peroxyacetic acid as antimicrobial intervention for controlling *Escherichia coli* O157:H7 on beef trimmings. LWT Food Sci Technol 65:868–873

Montville TJ, Matthews KR (2013) Physiology, growth and inhibition of microbes in food. In: Doyle MP, Buchanan R (eds) Food microbiology: fundamentals and frontiers. ASM Press, Washington, DC

Mosqueda-Melgar J, Raybaudi-Massilia RM, Martin-Belloso O (2008) Non-thermal pasteurization of fruit juices by combining high-intensity pulsed electric fields with natural antimicrobials. Innovative Food Sci Emerg Technol 9:328–340

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:132–201

Ortega E, Linares L, Adriani C, Leotta G (2014) Carnicerías Saludables. Instituto de Promoción de la Carne Vacuna Argentina. http://www.ipcva.com.ar/files/manualcarniceros.pdf

Oteiza JM, Giannuzzi L, Zaritzky N (2010) Ultraviolet treatment of orange juice to inactivate *E. coli* O157:H7 as affected by native microflora. Food Bioprocess Technol 3:603–614

Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM (2013) Attribution of foodborne illnesses, hospitalization, and deaths to food commodities by using outbreaks data, United States, 1998-2008. Emerg Infect Dis 19:407–415

Park IK, Kang DH (2013) Effect of electropermeabilization by ohmic heating for inactivation of *Escherichia coli* O157:H7, *Salmonella enterica* Serovar Typhimurium, and *Listeria monocytogenes* in buffered peptone water and apple juice. Appl Environ Microbiol 9:7122–7129

Peresi JTM, Castanheira-Almeida IAZ, Ibelli-Vaz TM, Tavanelli-Hernandes R, Carvalho-Teixeira IS, Lima-Silva SI, Silva-Graciano RA, dos Santos LF (2016) Search for diarrheagenic *Escherichia coli* in raw kibbe samples reveals the presence of Shiga toxin-producing strains. Food Control 63:165–170

Pradel N, Livrelli V, De Champs C, Palcoux JB, Reynaud A, Scheutz F, Sirot J, Joly B, Forestier C (2000) Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. J Clin Microbiol 38:1023–1031

Rajkovic A, Smigic N, Devlieghere F (2010) Contemporary strategies in combating microbial contamination in food chain. Int J Food Microbiol 141:29–42

Rangel-Vargas E, Gómez-Aldapa CA, Torres-Vitela M, Villarruel-López A, Gordillo-Martínez AJ, Castro-Rosas J (2015) Presence and correlation of some enteric indicator bacteria, diarrheagenic *Escherichia coli* pathotypes, and *Salmonella* serotypes in alfalfa sprouts from local retail markets in Pachuca, Mexico. J Food Prot 78:609–614

Rivas M, Chinen I, Miliwebsky E, Galli L, Repetto HA, Masana M (2011) Epidemiology of Argentinean STEC. In: Walk S, Feng P (eds) Bacterial population genetics: a tribute to Thomas S. Whittam. ASM Press, Washington, DC, pp 109–132

Rivero M, Passucci J, Lucchesi P, Signorini M, Alconcher L, Rodríguez E, Rocha Martín V, Meneguzzi B, San Juan F, Ballesteros B, Tarabla H (2013) Epidemiology of hemolytic uremic syndrome in two regions of Buenos Aires province. Medicina (B Aires) 73:127–135

Rodríguez R (2006) Calidad integral de alimentos y ecología microbiana. Academia Nacional de Agronomía y Veterinaria T LX. pp 75–131. http://sedici.unlp.edu.ar/bitstream/handle/10915/29129/Documento_completo.pdf?sequence=1

Signorini ML, Fizzo LS (2009) Modelo de contaminación cruzada por *Escherichia coli* verocitotoxigénica durante la elaboración de hamburguesas caseras y evaluación cuantitativa de riesgos. Rev Argent Microbiol 41:237–244

Signorini ML, Tarabla HD (2009) Quantitative risk assessment for verocytotoxigenic *Escherichia coli* in ground beef hamburgers in Argentina. Int J Food Microbiol 132:153–161

Signorini ML, Tarabla HD (2010) Interventions to reduce verocytotoxigenic *Escherichia coli* in ground beef in Argentina: a simulation study. Prev Vet Med 94:36–42

Silman AJ, Macfarlane GJ (2002) Epidemiological studies. Cambridge University Press, Cambridge

Smith BA, Fazil A, Lammerding AM (2013) A risk assessment model for *Escherichia coli* O157:H7 in ground beef and beef cuts in Canada: evaluating the effects of interventions. Food Control 29:364–381

Srednik ME, Rumi MV, Bentancor A (2014) Inocuidad de carne molida y presencia de cepas *Escherichia coli* causantes de lesiones de adherencia y esfacelación. InVet 15:123–130

Tawema P, Han J, Vu KD, Salmieri S, Lacroix M (2016) Antimicrobial effects of combined UV-C or gamma radiation with natural antimicrobial formulations against *Listeria monocytogenes*, *Escherichia coli* O157: H7, and total yeasts/molds in fresh cut cauliflower. LWT Food Sci Technol 65:451–456

Thomas MK, Majowicz SE, Sockett PN, Fazil A, Pollari F, Doré K, Flint JA, Edgeet VL (2006) Estimated numbers of community cases of illness due to *Salmonella*, *Campylobacter* and verotoxigenic *Escherichia coli*: pathogen-specific community rates. Can J Infect Dis Med Microbiol 17:229–234

Torso LM, Voorhees RE, Forest SA, Gordon AZ, Silvestri SA, Kissler B, Schlackman J, Sandt CH, Toma P, Bachert J, Mertz KJ, Harrison LH (2015) *Escherichia coli* O157:H7 outbreak associated with restaurant beef grinding. J Food Prot 78:1272–1279

Trinetta V, Vaidya N, Linton R, Morgan M (2011) A comparative study on the effectiveness of chlorine dioxide gas, ozone gas and e-beam irradiation treatments for inactivation of pathogens inoculated onto tomato, cantaloupe and lettuce seeds. Int J Food Microbiol 146:203–206

USDA (2013) Timeline of events related to *Escherichia coli* O157:H7. http://www.fsis.usda.gov/wps/portal/fsis/topics/regulatory-compliance/haccp/updates-and-memos/timeline-of-events-related-to-e-coli-o157h7/e-coli-timeline

USDA (2016) US Food Safety and Inspection Service. Imported meat passed for entry in the U.S. by country USDA livestock, poultry & grain market news. https://www.ams.usda.gov/mnreports/lswimpe.pdf

USDA MLG 5.09 (2015) Detection, isolation and identification of *Escherichia coli* O157:H7 from meat products and carcass and environmental sponges. http://www.fsis.usda.gov/wps/wcm/connect/51507fdb-dded-47f7-862d-ad80c3ee1738/MLG-5.pdf?MOD=AJPERES

USDA MLG 5B.05 (2015) Detection and isolation of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) from meat products and carcass and environmental sponges. http://www.fsis.usda.gov/wps/wcm/connect/7ffc02b5-3d33-4a79-b50c-81f208893204/MLG-5B.pdf?MOD=AJPERES

Voetsch AC, Kennedy MH, Keene WE, Smith KE, Rabatsky-Her T, Zansky S, Thomas SM, Mohle-Boetani J, Sparling PH, McGavern MB, Mead PS (2007) Risk factors for sporadic Shiga toxin-producing *Escherichia coli* O157 infections in FoodNet sites, 1999-2000. Epidemiol Infect 135:993–1000

Wang F, Yang Q, Kase JA, Meng J, Clotilde LM, Lin A, Ge B (2013) Current trends in detecting non-O157 Shiga toxin-producing *Escherichia coli* in food. Foodborne Pathog Dis 10:665–677

Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, Monson T, Koschmann C, Davis JP (2009) Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. Clin Infect Dis 48:1079–1086

Xia X, Meng J, McDermott PF, Ayers S, Blickenstaff K, Tran TT, Abbott J, Zheng J, Zhao S (2010) Presence and characterization of Shiga toxin-producing *Escherichia coli* and other potentially diarrheagenic *E. coli* strains in retail meats. Appl Environ Microbiol 76:1709–1717

Zhang C, Lu Z, Li Y, Shang Y, Zhang G, Cao W (2011) Reduction of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis on mung bean seeds and sprouts by slightly acidic electrolyzed water. Food Control 22:792–796

Zhou Y, Karwe MV, Matthews KR (2016) Differences in inactivation of *Escherichia coli* O157:H7 strains in ground beef following repeated high pressure processing treatments and cold storage. Food Microbiol 59:7–12

Chapter 9
Extra-Intestinal *Escherichia coli* (Uropathogenic *E. coli* and Avian Pathogenic *E. coli*)

Janaína L. Leite, Thaís C.G. Rojas, Renato P. Maluta, and Wanderley Dias de Silveira

Summary Extra-intestinal pathogenic *E. coli* (ExPEC) is the most common Gram-negative bacterial pathogen, and it is associated to many animal diseases. Within the ExPEC classification are the uropathogenic *Escherichia coli* strains that are able to cause pyelonephritis, prostatitis, and catheter-associated urinary tract infections (UTIs) as well as community-acquired and nosocomial UTIs in humans, accounting for substantial medical costs and morbidity and mortality worldwide. Many virulence factors, such as adhesins, toxins, capsule, serum resistance, and iron uptake systems, are linked to the pathogenicity presented by these strains. A zoonotic potential of ExPEC has been suggested due to a close relationship between ExPECs from human and avian origins. Avian pathogenic *Escherichia coli* is a group of ExPEC strains that cause several infectious processes termed colibacillosis, representing the major cause of losses in the poultry industry, resulting in decreased egg production, influencing the cost of treatment, and impacting carcasses condemnations and mortality. APEC strains can trigger localized and systemic forms of diseases which include omphalitis, cellulitis, swollen-head syndrome, salpingitis and peritonitis, coligranuloma, colisepticemia, and air sac disease. Some recent advances in ExPEC research have been done about multidrug resistance, prophylactic vaccines, new technologies, and therapies such as bio-conjugation, fusion of proteins, and the use of mannosides and pilicides. Also, in Latin America many studies have been realized about ExPEC strains such as antibiotic resistance, virulence factors, and their pathogenicity as well as the development of potential vaccines.

J.L. Leite • T.C.G. Rojas • R.P. Maluta • W.D. de Silveira (✉)
Bacterial Molecular Biology Laboratory, Department of Genetics, Evolution and Bioagents, University of Campinas (UNICAMP), São Paulo, Brazil
E-mail: jgarbim@unicamp.br; thaiscgrojas@gmail.com; maluta@gmail.com; wds@unicamp.br

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_9

1 Introduction to Extra-Intestinal Pathogenic *Escherichia coli*

Among all bacteria, *Escherichia coli* is probably, the most ubiquitous microorganism, being found in virtually all environments and associated with the majority of living beings. Most of *E. coli* strains are commensal microorganisms, but some strains can be pathogenic to humans and other hosts. Many reviews about pathogenic *E. coli* strains and their pathogenesis processes have been published (Kaper et al. 2004; Bien et al. 2012; Dale and Woodford 2015; Poolman and Wacker 2016) and are available for further details.

In humans, extra-intestinal pathogenic *E. coli* (ExPEC) is the most common Gram-negative bacterial pathogen. It is associated to many clinical diseases such as bacteremia (septicemic *E. coli*—SPEC), meningitis in neonates (neonatal meningitis *E. coli*—NEMEC), and up to 80 % of uncomplicated, community-acquired urinary tract infections (UTIs) (uropathogenic *E. coli*—UPEC) in all age groups (Russo and Johnson 2003; Kaper et al. 2004; Poolman and Wacker 2016). Also in humans, UPEC strains are able to cause pyelonephritis, prostatitis, and catheter-associated UTIs (Dale and Woodford 2015), as well as other infections such as those associated with abdominal and pelvic sources, including biliary infections, peritonitis, and pelvic inflammatory disease (Chen and Hsueh 2012; Sun et al. 2013; Sharma et al. 2014).

UPEC is the cause of community-acquired and nosocomial UTIs, accounting for substantial medical costs and morbidity and mortality worldwide (Russo and Johnson 2003). UTI is among the most common bacterial infections in humans and has been shown to be an independent risk factor for both, bladder cancer and renal cell carcinoma (Parker et al. 2004). The intestinal tract is an ExPEC reservoir of particular importance for women with recurrent UTIs (rUTIs). It is estimated that over 50 % of women will have an UTI in their lifetime; 25 % will then experience a second UTI, and 3 % will have a third UTI within 6 months of the initial infection (Foxman 2003). One explanation for recurrence, besides urinary tract malformations, is the fact that the UPEC reservoir for women; it is their own gastrointestinal tract. So, UPEC would continuously be reintroduced to the urinary tract, allowing subsequent infections to occur. Indeed, UPEC clones persist long term as commensals within the intestinal tract and can even be shared among family members and household pets (Murray 2004; Johnson et al. 2008). A diagnostic test that could identify potential carriers of UPEC; therefore, would be beneficial for medical practitioners to determine a course for prevention of rUTI. Identification of reservoirs of uropathogens could be used to identify the risk factors of populations and reduce disease transmission. Once carriers of UPEC are identified, the patients could be treated to eliminate the pathogen (Spurbeck et al. 2012).

E. coli strains, including UPEC, are normally classified according to the presence of O, K, and H antigens. The O antigen forms part of the *E. coli* lipopolysaccharide (LPS) (Stenutz et al. 2006); the K antigen is the *E. coli* capsule; and the H antigen is

the flagellum (Totsika et al. 2012). The distribution of ExPEC strains in their infection processes and hosts can be influenced by age and the site of infection. From 180 O serotypes currently described, only a subset of these are associated with the majority of ExPEC infections, with limited geographic variation (Stenutz et al. 2006). For humans, 10–12 O serotypes account for approximately 90 % of meningitis isolates and more than 60 % of bacteremia isolates (Cross et al. 1994). Serogroups O1, O2, O4, O6, O7, O8, O16, O18, O25, and O75 are the most frequent in UPEC strains (Totsika et al. 2012).

In UPEC pathogenesis, there are evidences that bacterial strains presenting K1 or O18 antigens encode more virulence-associated factors than other ExPEC isolates (Ewers et al. 2007). Flagella (H antigens) is related to the ascension of UPEC from the bladder into the kidneys (Hannan et al. 2012).

Regarding the association of certain UPEC serogroups to many virulence factors, such as adhesins (type 1 and P-fimbriae), toxins (hemolysin and cytotoxic necrotizing factor), capsule, serum resistance, and iron uptake systems (enterobactin, aerobactin), they are linked to the pathogenicity presented by these strains. In many cases, several of these virulence factors are located on mobile genetic elements named pathogenicity islands (Dale and Woodford 2015).

Alpha-hemolysin (HlyA) is a lipoprotein associated with upper UTIs, such as pyelonephritis. This secreted toxin forms pores in the cell membrane, leading to the lysis of eukaryotic cells, causing hemorrhage in a murine model of infection, inhibiting cytokine production, and promoting the exfoliation of bladder epithelial cells (Jonas et al. 1993; Dhakal and Mulvey 2012). Also, HlyA improves the ability of extra-intestinal pathogens like UPEC to cross over mucosal barriers to damage effector immune cells and to gain enhanced access to host nutrients and iron sources (Dhakal and Mulvey 2012).

Adhesins are adhesive molecules that allow bacteria to bind and recognize receptors on the surface of the host. They are the most important determinant of pathogenicity of UPEC, triggering host and bacterial cell signaling pathways, helping to deliver other bacterial products to host tissues, and promoting bacterial invasion (Mulvey 2002). Type 1 fimbria is important to UTIs by mediating adhesion to mannose-containing receptors on the uroepithelium and promoting the intracellular bacterial growth. P fimbriae are heteropolymeric fibers encoded by the *papA-K* gene operon and act on pathogenesis of ascending UTIs and pyelonephritis in humans (Lane and Mobley 2007). These fimbriae are responsible for triggering the production of cytokines and adhesion to mucosal and tissue matrix (Hedlund et al. 1999; Godaly et al. 2000). In renal transplanted patients, the P fimbriae are the most common virulence factor and *papG* class II allele is isolated from strains of patients with acute renal dysfunction. The S and F1C fimbriae act in the epithelial and endothelial cell lines from human urinary tract and kidney (Marre et al. 1990; Mulvey 2002). The S fimbria facilitates bacterial dissemination and is associated with *E. coli* strains that cause sepsis (SEPEC), NMEC, and ascending UTIs. The Dr fimbrial and afimbrial Afa adhesins of *E. coli* are associated with chronic and/or recurrent UTIs (rUTIs) (gestational

pyelonephritis and recurring cystitis) (Nowicki and Nowicki 2013; Zalewska-Piatek 2011). The autotransporter protein, antigen 43 (Ag43), has been related to the persistence of *E. coli* in the urinary tract, causing asymptomatic bacteriuria, cystitis, pyelonephritis, and biofilm formation (Ulett et al. 2007; Mabbett et al. 2009).

Other surface-exposed virulence factors include the capsule and the lipopolysaccharide (LPS). The capsule is a polysaccharide structure covering and protecting the bacterium against phagocytic engulfment and complement-mediated bactericidal effect of the host immune system. The LPS is an integral component of the cell wall of Gram-negative bacteria, and it is known to activate host response and to induce nitric oxide and cytokine production (Eder et al. 2009). In UPEC, LPS is related to the pro-inflammatory response in uncomplicated UTIs. The receptor TLR4 is expressed in both renal epithelia and pelvis, suggesting that the ascending infection due to *E. coli* may stimulate the innate immune response associated with the acute allograft injury in patients with UTIs (Wolfs et al. 2002; Samuelsson et al. 2004). Flagellated UPEC strains cause up to 70–90 % of all UTIs, and their pathogenesis involves contact between the bacteria and epithelial cell surface of the urinary tract. The pyelonephritis-associated *E. coli* strains may invade renal collecting duct cells through flagellin, acting as an invasin in this process (Pichon et al. 2009). The Cytotoxic Necrotizing Factor 1 (CNF1) produced by one-third of all pyelonephritis strains is related to kidney invasion (De Rycke and Milon 1999; Landraud et al. 2000). This protein is secreted by *E. coli*, stimulating actin stress fibers formation and membrane ruffle formation, in a Rho GTPase-dependent manner and resulting in the entry of bacteria into the cells. Studies in vitro and in vivo have showed that CNF1 acts with polymorphonuclear phagocytosis triggering exfoliation and apoptotic death of bladder epithelial cells facilitating the access to underlying tissue (Fiorentini et al. 1997; Mills et al. 2000). Secreted autotransporter toxin (SAT) is produced by pyelonephritis *E. coli* strains and has a toxic activity against cell lines of bladder or kidney origin, and it has been related to pathogenesis of UTIs (Guyer et al. 2000; 2002).

A phylogenetic tree based on Sequence Type (ST) demonstrated that a close relationship exist between ExPECs from human and avian origins (Moulin-Schouleur et al. 2007). Patterns obtained by pulsed-field gel electrophoresis (PFGE) also revealed similarities between APEC (avian pathogenic *E. coli*) and human ExPEC, displaying indistinguishable patterns from human UTI and *E. coli* from chicken retail (Vincent et al. 2010). Some of the most studied genes of ExPEC virulence factors are cited in the Table 9.1. The same kind of results were also obtained by a study performed with a Brazilian ExPEC collection, demonstrating that ST359 strains from human and avian hosts were somewhat related (Maluta et al. 2014). Additionally, it was demonstrated that some human ExPEC strains were virulent for chickens (Moulin-Schouleur et al. 2007) and that APEC plasmids can contribute to the uropathogenicity of *E. coli* (Skyberg et al. 2006). Furthermore, it is well documented that APEC strains can act as reservoirs for genes and plasmids, that encodes for virulence genes and antimicrobial resistance. It has been demonstrated that 92 % of poultry meat samples tested in a study conducted by Johnson et al. (2005a) were

Table 9.1 Virulence genes frequently studied in extra-intestinal pathogenic *Escherichia coli* (UPEC and APEC strains)

Gene	Function
afa	Adhesin
bmaE	Adhesin
cnf1	Toxin
csgA	Adhesin
cvaC	Protectin
ecpA	Fimbriae
fimH	Adhesin
fyuA	Iron-acquisition
hlyA	Toxin
hlyF	Toxin
ibeA	Invasion
ireA	Iron-acquisition
iroN	Iron-acquisition
Iss	Serum resistance
iutA	Iron-acquisition
kpsMTII	Capsular synthesis
malX	Pathogenicity island
ompT	Outer membrane protein
papC	Adhesin
papG	Adhesin
sfa	Adhesin
sitD	Iron-acquisition
traT	Serum resistance
tsh	Serine protease autotransporter
vat	Toxin

contaminated with *E. coli*, which possessed virulence factors associated with ExPEC. Because of the studies on APEC zoonotic risk, the Centers for Disease Control and Prevention issued a report alerting for the zoonotic potential of ExPEC and the possible transmission via chicken meat (Vincent et al. 2010; Bergeron et al. 2012).

Avian colibacillosis is defined as an infection caused by APEC or an association of APEC and other factors, such as virus or stress caused by inappropriate husbandry practices. Colibacillosis, in all its forms, represents the major cause of losses in poultry industry, due to decreased production, including egg production, influencing the cost of treatment, and impacting carcasses condemnations and mortality (Zanella et al. 2000; Gross 1994). It is estimated that *E. coli* infections mortality rate ranges from 1 to 10 % in chickens, with elevated rates in broilers (Zanella et al. 2000).

The natural route of infection, which also represents a common way to introduce new serotypes into poultry flocks, seems to be oral, due to the ingestion of contaminated feed and water. Another possible route is the inhalation of dust into the poultry

houses that can contain up to 10^6 colony-forming units of *E. coli* per gram (Harry 1964; Dziva and Stevens 2008).

The clinical signs associated with APEC infection are depression, fever, yellowish or greenish droppings, and lesions of internal organs (Fig. 9.1). APEC infections can be systemic or localized, and the disease name often refers to the affected tissue. Localized forms of colibacillosis are: omphalitis, cellulitis, swollen-head syndrome, salpingitis, and peritonitis. Systemic forms of APEC infections include: coligranuloma, colisepticemia, and air sac disease (Barnes et al. 2008).

Omphalitis/yolk sac infection is the inflammation of the umbilicus (and the yolk sac in birds). The infection can occur by the contamination of the eggs if the hen has salpingitis, via artificial insemination or at laying, and can be lethal to embryo and early chick. Pathogenic *E. coli* strains are more frequently found on the intestinal tract of newly hatched chicks than in the eggs that they hatched, indicating that the most important contamination of pathogenic strain is due to feces, for both the eggs and the chicks (Pourbakhsh et al. 1997). Inflammation of the oviduct, caused by *E. coli* ascending from cloaca, results in salpingitis, which is responsible for decreased in egg production and can frequently result in death. Coliform peritonitis is frequently the result of the spread of the coliforms from infected oviduct into body cavity, resulting in extensive inflammation, exudation, and positive cultures (Barnes et al. 2008).

Cellulitis is characterized by the inflammation of the subcutaneous tissues and the presence of fibrino-necrotic plaques, resulting in a significant disease problem

Fig. 9.1 Chicken infected with *Escherichia coli* strain SCI-07 (APEC), showing the liver points of lesions (*black arrows*). This chicken of 1-day-old was infected into the right thoracic air sac. Seventh days' post infection

in broiler chickens because the rejection of the carcasses at processing (Ngeleka et al. 1996; Barnes et al. 2008).

Swollen-head syndrome is an acute cellulitis of the periorbital and adjacent subcutaneous tissues of the head, caused by inflammatory exudate below the skin. APEC contamination usually occurs after upper respiratory viral infections by avian pneumovirus or bronchitis virus, by the contamination of the conjunctiva or the mucous membranes of the sinuses or nasal cavity (Droual and Woolcock 1994; Nakamura et al. 1997).

Colisepticemia is the result of a systemic infection determined by the presence of pathogenic *E. coli* into the blood stream. Severity of the disease is determined by the virulence of the bacteria, host defense system conditions, and the extension of the inflammation (Pourbakhsh et al. 1997; Barnes et al. 2008).

Respiratory systemic infection, also known as air sac disease, is caused by the infection of respiratory mucosae by APEC. Some predisposing agents such as bronchitis virus, Newcastle disease virus, mycoplasmas infection, and ammonia can worsen the disease (Al-Ankari et al. 2001; Jirjis et al. 2004). Lesions can reach trachea, lungs, air sacs, pericardial sac, and peritoneal cavities preceding bacteremia (Barnes et al. 2008). Another form of systemic colibacillosis is coligranuloma that is determined by the presence of multiple granulomas in liver, ceca, duodenum, and mesentery.

Colibacillosis is a serious threat to the poultry industry worldwide, especially nowadays where the chicken meat is the most consumed animal protein by humans (Qabajah et al. 2014). The biggest producers of chicken and turkey meat in the world are the United States, China, and Brazil, being this last, the bigger exporter. Additionally, Brazil is one of the largest egg producers (ABPA 2015). The disease has a global nature, and the pathogen display an extensive genetic diversity, which can be outlined by environmental factors such as climate, host, exposure to antimicrobials, and microenvironmental different pressures (Gyles 2008; Qabajah et al. 2014).

The vertical transmission can occur by egg deposition and may cause high chick mortality (Petersen et al. 2006). Despite this, the most efficient egg *E. coli* transmission is via feces deposition on the egg surface, whereas coliforms may penetrate the shell and membranes (Barnes et al. 2008).

In healthy birds, it is estimated that up to 10–15% of the intestinal coliforms belong to potentially pathogenic serogroups. Then, the intestinal tract of healthy chickens are reservoirs of virulence and drug-resistance genetic traits (Nógrády et al. 2006; Gyles 2008).

Regarding the virulence factors to be present in APEC strains, some serogroups are prevalent including O1, O8, O18, O35, O36, O78, O111, which reinforces the similarities among APEC and UPEC (Ewers et al. 2007; Maluta et al. 2014).

In APEC infections, several studies demonstrated that a combination of *E. coli* virulence factors is responsible for the pathogenesis processes. A previous study proposed that different combinations of the genes encoding for temperature-sensitive hemagglutinin (*tsh*), for P (*pap*) and F1 (*pil*) fimbriae, and for aerobactin synthesis (*iuc*) were enough to distinguish different APEC pathotypes, been *tsh*/ *pil*/*iuc* and

tsh/*pap*/*iuc* the most common combination (Ngeleka et al. 2002). The use of multiplex polymerase chain reaction (PCR) was proposed by different studies as the way to identify common virulence traits, including the ColV plasmid-borne traits, which are widely associated with APEC virulence (Johnson et al. 2006; Tivendale et al. 2009; Mellata et al. 2010). Using this methodology, strains are classified as APEC if they harbor at least four of the following eight genes: P fimbria (*papC*), aerobactin (*iucD*), iron repressible protein (*irp2*), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter protein (*vat*), enteroaggregative toxin (*astA*), increased serum survival protein (*iss*), and colicin plasmid operon genes (*cva*/*cvi*). Another study demonstrated that the detection of genes encoding iron acquisition systems (*iutA*, *sitA*), P fimbriae (*F11*), sugar metabolism (*frzorf4*), O-antigen O78, and type six secretion system-T6SS (*aec26*, *aec4*), would identify 70.2 % of APEC strains and should be used for diagnostic purposes (Schouler et al. 2012).

Biological and genetic characteristics and pathogenicity of APEC strains suffering from omphalitis, septicemia, and swollen-head syndrome have been studied by our group. Characteristics such as toxins and siderophores production, adhesion and invasion in eukaryotic cells, in the presence or absence of D-mannose, antibiotic and serum resistance, flagellum and fimbriae expression were analyzed (da Silveira et al. 2002, 2003; Stehling et al. 2008; de Campos et al. 2005; Maluta et al. 2014). The analysis of Type 6 Secretion System of proteins produced by *E. coli* and the null mutant construction of some APEC strains was also studied by our group (de Pace et al. 2010, 2011; de Paiva et al. 2015; Verma et al. 2015; Pilatti et al. 2016). A study proposing a new classification within APEC strains in pathotypes or subpathotypes, according to specific infection syndrome of host, was demonstrated (Maturana et al. 2011). Furthermore, the genetic analysis of some APEC strains from our collection by genome sequencing was done, enabling a more complete study of virulence factors of these strains (Rojas et al. 2012, 2013).

2 Recent Advances in ExPEC Research

Although there are no consistent data on Latin America cases of UTIs, the majority of uncomplicated infections, rUTIs, cases of pyelonephritis and around one-third of catheter-associated infections in the United States are caused by ExPEC (Poolman and Wacker 2016). A study in 2014 reported that 80,089 hospital admissions in 19 US hospitals between 2007 and 2010, 47 % were urinary tract diseases, being 52 % due to *E. coli* infection (Edelsberg et al. 2014).

The worldwide emergence of the multidrug-resistant (MDR) *E. coli* sequence type O25b:ST131 clone represents a major challenge for prevention and management of *E. coli* infections (Mathers et al. 2015). However, there is no prophylactic vaccine against ExPEC yet, due to many technical limitations and the seek for a multivalent vaccine would be very complex (Poolman and Wacker 2016) and would have a very high cost.

Bio-conjugation refers to the biosynthesis of polysaccharide and carrier proteins within *E. coli* cells and their subsequent in vivo coupling by the use of the oligosaccharyltransferase PglB from the N-linked protein glycosylation system originally identified in *Campylobacter jejuni* and subsequently transferred to *E. coli* (Feldman et al. 2005). As a result of this technology, a 4-valent prototype *E. coli* bioconjugate vaccine (EcoXyn-4V; GlycoVaxyn) was proposed and evaluated in a phase 1 study, with results were expected to be valued at the end of 2015 (Poolman and Wacker 2016).

Vaccine manufacturers also need to consider whether a candidate ExPEC vaccine should be used for general, for a specific indication (as different vaccines for prevention of UTI and bacteremia), or for strain specificity (as strains are highly antibiotic resistant). According to recent discoveries, the O serotype 25b, is strongly associated with ST131, and will need to be included in any ExPEC commercial vaccine (Poolman and Wacker 2016).

Several vaccination approaches have been explored, including heat-killed whole bacteria, bacterial cell extracts, and purified UPEC-associated virulence factors as antigens. The use of a vaginal suppository (Solco Urovac) loaded with ten heat-killed strains of uropathogenic bacteria (six *E. coli* strains plus one strain each of *Proteus mirabilis, Morganella morganii*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*) for vaccination of women presenting rUTI was promising in reducing the incidence of UTI caused by *E. coli* in sexually active women between 20 and 50 years of age (Uehling et al. 2003; Hopkins et al. 2007).

Researchers have also specifically targeted the FimH adhesin by use of soluble receptor analogues, or mannosides, that act as antiadhesive compounds. These molecules bind FimH and prevent it from interacting with host receptors (Han et al. 2011). In a murine UTI model of infection, mannoside derivatives work prophylactically, preventing bacterial invasion into bladder tissue (Cusumano et al. 2011). They can also be used to treat established and catheter-associated infections, acting synergistically with standard antibiotic treatments to reduce UPEC titers within the urinary tract of infected mice (Guiton et al. 2012). Both mannosides and pilicides have exciting potential as future therapies for the treatment of uncomplicated cystitis and rUTI, and both types of reagents may help circumvent the rising tide of antibiotic-resistant organisms (Barber et al. 2013).

In 2015, the Fusion Protein Technology was demonstrated, based on incorporation of target antigens from one or more pathogen (MrpH P fimbria of *P. mirabilis* and FimH adhesin of UPEC), in just one molecule to obstruct different virulence mechanisms or prevent infection caused by different pathogens (Olsen et al. 2001; Skeiky et al. 2002; Cuadros et al. 2004) using BALB/c mice via intranasal immunization (Habibi et al. 2015). A strong humoral and cellular immune responses was induced in vaccinated mice eliminating both superficial and intracellular reservoirs of UPEC and *P. mirabilis*, suggesting that vaccination generated a long-lived memory cells (Brumbaugh et al. 2013).

The yersiniabactin receptor, whose gene is highly represented among the genomes of pyelonephritis (94 %) and cystitis (87 %) strains (Johnson et al. 2005b),

it is a strong candidate antigen for inclusion in a multivalent UTI vaccine. Novel antimicrobial agents or vaccines that target yersiniabactin iron transport or other pathogen-associated nutrient acquisition systems may provide an effective strategy to combat the rising surge of antibiotic-resistant common infections (Brumbaugh et al. 2015). In a mutagenesis experiment, the authors studied the yersiniabactin system as a UPEC virulence factor during cystitis and pyelonephritis, a fitness factor during bacteremia and the surface-accessible target of the FyuA vaccine. Also, the transcriptome sequencing (RNA-seq) analysis of *E. coli* in urine from women with cystitis, was used to demonstrate that encoding genes of iron acquisition systems, including the yersiniabactin system, are highly expressed by bacteria during natural uncomplicated UTI and cystitis (Brumbaugh et al. 2015).

Research in APEC-pathogenicity process, related to new genes discovered, gained great impulse in recent years due to the availability and popularity of sequencing methodologies. Genomes added to public database also increased, allowing the search for new virulence factors that can be employed on APEC characterization (Verma et al. 2015; de Paiva et al. 2015; Rojas et al. 2012; 2013).

Because the extensive use of tetracyclines, fluoroquinolones, and sulfonamides to control APEC infections and the significant growth of the poultry industry worldwide, it has been observed an increase in the level of avian *E. coli* resistance to antibiotics (Zhao et al. 2005). The main concern about avian *E. coli* to MDR is not only because of the inefficacy in treatments, which has been observed (Barnes et al. 2008), but mainly due to the increase of antibiotic resistance in human ExPEC strains. The acquisition of antibiotic resistance traits is, at least in part, the result of the incorporation of transferable mobile genetics elements (transposons, integrons, and plasmids) directly from avian *E. coli*, by the direct contact or food consumption (Manges and Johnson 2012), or from other Gram-negative bacteria that have acquired those elements from avian *E coli* on the intestinal tract from chicks (Yang et al. 2004).

3 ExPEC in Latin America

A recent study in Southern Brazil showed a higher antimicrobial resistance profiles in ExPEC compared to commensal isolates (approximately 1/3 of strains resistant to fluoroquinolones and trimethoprim-sulfamethoxazole) compared to other studies (Cyoia et al. 2015). In this work, the authors suggested the necessity of research on virulence factors of ExPEC to clarify which genes would be ideal virulence markers (Cyoia et al. 2015).

In Mexico, it was reported higher antibiotic resistance than that observed in developed countries, as well as that reported in other Latin American countries (Molina-López et al. 2011; Amabile-Cuevas 2010). In these studies, approximately 30 % of UPEC community-acquired isolates from Mexico City, presented MDR phenotypes, resulting in serious infections in ambulatory population, which has a few available options to treat UTIs. Indeed, some *E. coli* O25-ST131 isolates were

found (Molina-López et al. 2011), and this clone has been disseminated worldwide (Nicolas-Chanoine et al. 2008).

In Brazil, the use of antimicrobial agents (antibiotics) as growth promoters is prohibited nowadays, but was extensively used until recent years in poultry industry. A recent study conducted by Koga et al. 2015 analyzed the frequency of antimicrobial resistance among *E. coli* strains isolated from commercial chicken carcasses in Parana State, Brazil, in 2007 and 2013. They detected an increase in frequency of tetracyclines, β-lactams, and quinolones in the carcass isolated in 2013, when compared to those collected in 2007, suggesting that there was a significant increase in the use of tetracyclines, cephalosporins (β-lactams), and quinolones in poultry production. Despite the necessity, up to date there is no vaccine against APEC that has proved to be effective for commercial production. The studies about APEC vaccines englobed different kinds of immunization, including inactivated strains, subunits, and live attenuated vaccines. Several attempts to develop an effective vaccine against APEC reached some success, which can be measured by its safety and protection provided (Ghunaim et al. 2014). However, to have an efficient APEC vaccine, it is crucial that the protection occurs against very heterogeneous serogroups. The studies that tested inactivated vaccine failed in this regard, providing only homologous protection. Unlike, the studies with live attenuated or conserved subunit vaccine had promising results against heterologous, but come across to others difficult aspects, such as the necessity of repeated administration of the vaccine with a strong adjuvant, which makes this kind of vaccine less competitive for commercial use than the live vaccines that can be administered as aerosol. Thus, considering the scenario described above, further investigations, with large-scale studies simulating the poultry industry conditions are need to develop effective APEC vaccines (Ghunaim et al. 2014).

References

Al-Ankari AR, Bradbury JM, Naylor CJ et al (2001) Avian pneumovirus infection in broiler chicks inoculated with *Escherichia coli* at different time intervals. Avian Pathol 30:257–267

Amabile-Cuevas C (2010) Antibiotic resistance in Mexico: a brief overview of the current status and its causes. J Infect Dev Ctries 4:126–131

ABPA Annual Report (2015) Associação Brasileira de Proteína Animal. abpa-br.com.br/files/RelatorioAnual_UBABEF_2015_DIGITAL.pdf. Accessed 15 April 2016

Barber AE, Norton JP, Spivak AM et al (2013) Urinary tract infections: current and emerging management strategies. Clin Infect Dis 57:719–724

Barnes H, Nolan L, Vaillancourt J (2008) Colibacillosis. In: Saif Y (ed) Diseases of poultry, 12th edn. Blackwell, Ames

Bergeron CR, Prussing C, Boerlin P et al (2012) Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada. Emerg Infect Dis 18:415–421

Bien J, Sokolova O, Bozko P (2012) Role of uropathogenic *Escherichia coli* virulence factors in development of urinary tract infection and kidney damage. Int J Nephrol 2012:681473

Brumbaugh AR, Smith SN, Mobley HLT (2013) Immunization with the yersiniabactin receptor, *fyuA*, protects against pyelonephritis in a murine model of urinary tract infection. Infect Immun 81:3309–3316

Brumbaugh AR, Smith SN, Subashchandrabose S et al (2015) Blocking yersiniabactin import attenuates extraintestinal pathogenic *Escherichia coli* in cystitis and pyelonephritis and represents a novel target to prevent urinary tract infection. Infect Immun 83:1443–1450

Cross A, Artenstein A, Que J et al (1994) Safety and immunogenicity of a polyvalent *Escherichia coli* vaccine in human volunteers. J Infect Dis 170:834–840

Cuadros C, Lopez-Hernandez FJ, Lucia A et al (2004) Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. Infect Immun 72:2810–2816

Cusumano CK, Pinkner JS, Han Z et al (2011) Treatment and prevention of urinary tract infection with orally active FimH inhibitors. Sci Transl Med 3:109–115

Cyoia PS, Rodrigues GR, Nishio EK et al (2015) Presence of virulence genes and pathogenicity islands in extraintestinal pathogenic *Escherichia coli* isolates from Brazil. J Infect Dev Ctries 9:1068–1075

da Silveira WD, Ferreira A, Brocchi M et al (2002) Biological characteristics and pathogenicity of avian *Escherichia coli* strains. Vet Microbiol 85:47–53

da Silveira WD, Lancellotti M, Ferreira A et al (2003) Determination of the clonal structure of avian *Escherichia coli* strains by isoenzyme and ribotyping analysis. J Vet Med B Infect Dis Vet Public Health 50:63–69

Dale AP, Woodford N (2015) Extra-intestinal pathogenic *Escherichia coli* (ExPEC): disease, carriage and clones. J Infect 71:615–626

de Campos TA, Stehling EG, Ferreira A et al (2005) Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian *Escherichia coli* strains. Vet Microbiol 106:275–285

de Pace F, Nakazato G, Pacheco A et al (2010) The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infect Immun 78:4990–4998

de Pace F, Boldrin de Paiva J, Nakazato G et al (2011) Characterization of IcmF of the type VI secretion system in an avian pathogenic *Escherichia coli* (APEC) strain. Microbiology 157:2954–2962

de Paiva JB, Leite JL, Da Silva LPM et al (2015) Influence of the major nitrite transporter NirC on the virulence of a swollen head syndrome avian pathogenic *E. coli* (APEC) strain. Vet Microbiol 175:123–131

de Rycke J, Milon A, Oswald E (1999) Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. Vet Res 30:221–33

Dhakal BK, Mulvey MA (2012) The UPEC pore-forming toxin alpha-hemolysin triggers proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways. Cell Host Microbe 11:58–69

Droual R, Woolcock PR (1994) Swollen head syndrome associated with *E. coli* and infectious bronchitis virus in the Central Valley of California. Avian Pathol 23:733–742

Dziva F, Stevens MP (2008) Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathol 37:355–366

Edelsberg J, Weycker D, Barron R, Li X, Wu H, Oster G et al (2014) Prevalence of antibiotic resistance in US hospitals. Diagn Microbiol Infect Dis 78:255–262

Eder K, Vizler C, Kusz E et al (2009) The role of lipopolysaccharide moieties in macrophage response to *Escherichia coli*. Biochem Biophys Res Commun 389:46–51

Ewers C, Li G, Wilking H et al (2007) Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int J Med Microbiol 297:163–176

Feldman MF, Wacker M, Hernandez M et al (2005) Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. Proc Natl Acad Sci U S A 102:3016–3021

Fiorentini C, Fabbri A, Matarrese P et al (1997) Hinderance of apoptosis and phagocytic behaviour induced by *Escherichia coli* cytotoxic necrotizing factor 1: two related activities in epithelial cells. Biochem Biophys Res Commun 241:341–346

Foxman B (2003) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Dis Mon 49:53–70

Ghunaim H, Abu-Madi MA, Kariyawasam S (2014) Advances in vaccination against avian pathogenic *Escherichia coli* respiratory disease: potentials and limitations. Vet Microbiol 172:13–22

Godaly G, Bergsten G, Frendéus B et al (2000) Innate defences and resistance to gram negative mucosal infection. In: Genes and proteins underlying microbial urinary tract virulence. Kluwer Academic, Boston, pp 9–24

Gross WB (1994) Diseases due to *Escherichia coli* in poultry. In: Gyles CL (ed) *Escherichia coli* in domestic animals and humans. CAB International, Wallingford

Guiton PS, Cusumano CK, Kline KA et al (2012) Combinatorial small-molecule therapy prevents uropathogenic *Escherichia coli* catheter-associated urinary tract infections in mice. Antimicrob Agents Chemother 56:4738–4745

Guyer DM, Henderson IR, Nataro JP et al (2000) Identification of *sat*, an autotransporter toxin produced by uropathogenic *Escherichia coli*. Mol Microbiol 38:53–66

Guyer DM, Radulovic S, Jones FE et al (2002) Sat, the secreted autotransporter toxin of uropathogenic *Escherichia coli*, is a vacuolating cytotoxin for bladder and kidney epithelial cells. Infect Immun 70:4539–4546

Gyles CL (2008) Antimicrobial resistance in selected bacteria from poultry. Anim Health Res Rev 9:149–158

Habibi M, Asadi Karam MR, Shokrgozar MA et al (2015) Intranasal immunization with fusion protein MrpH.FimH and MPL adjuvant confers protection against urinary tract infections caused by uropathogenic *Escherichia coli* and Proteus mirabilis. Mol Immunol 64:285–294

Han Z, Pinkner JS, Ford B et al (2011) Structure-based drug design and optimization of mannoside bacterial FimH antagonists. J Med Chem 53:4779–4792

Hannan TJ, Totsika M, Mansfield KJ et al (2012) Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection. FEMS Microbiol Rev 36:616–648

Harry E (1964) The survival of *E. coli* in the dust of poultry houses. Vet Rec 76:466–470

Hedlund M, Wachtler C, Johansson E et al (1999) P fimbriae-dependent, lipopolysaccharide-independent activation of epithelial cytokine responses. Mol Microbiol 33:693–703

Hopkins WJ, Elkahwaji J, Beierle LM et al (2007) Vaginal mucosal vaccine for recurrent urinary tract infections in women: results of a phase 2 clinical trial. J Urol 177:1349–1353

Jirjis FF, Noll SL, Halvorson DA et al (2004) Effects of bacterial coinfection on the pathogenesis of avian pneumovirus infection in turkeys. Avian Dis 48:34–49

Johnson JR, Kuskowski MA, Gajewski A et al (2005a) Extended virulence genotypes and phylogenetic background of *Escherichia coli* isolates from patients with cystitis, pyelonephritis, or prostatitis. J Infect Dis 191:46–50

Johnson JR, Kuskowski MA, Smith K et al (2005b) Antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* in retail foods. J Infect Dis 191:1040–1049

Johnson TJ, Johnson SJ, Nolan LK (2006) Complete DNA sequence of a ColBM plasmid from avian pathogenic *Escherichia coli* suggests that it evolved from closely related ColV virulence plasmids. J Bacteriol 188:5975–5983

Johnson JR, Clabots C, Kuskowski MA (2008) Multiple-host sharing, long-term persistence, and virulence of *Escherichia coli* clones from human and animal household members. J Clin Microbiol 46:4078–4082

Jonas D, Schultheis B, Klas C et al (1993) Cytocidal effects of *Escherichia coli* hemolysin on human T lymphocytes. Infect Immun 61:1715–1721

Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140

Koga VL, Rodrigues GR, Scandorieiro S et al (2015) Evaluation of the antibiotic resistance and virulence of *Escherichia coli* strains isolated from chicken carcasses in 2007 and 2013 from Paraná, Brazil. Foodborne Pathog Dis 12:479–485

Landraud L, Gauthier M, Fosse T et al (2000) Frequency of *Escherichia coli* strains producing the cytotoxic necrotizing factor (CNF1) in nosocomial urinary tract infections. Lett Appl Microbiol 30:213–216

Lane MC, Mobley HLT (2007) Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic *Escherichia coli* (UPEC) in the mammalian kidney. Kidney Int 72:19–25

Mabbett AN, Ulett GC, Watts RE et al (2009) Virulence properties of asymptomatic bacteriuria *Escherichia coli*. Int J Med Microbiol 299:53–63

Maluta RP, Gatti MSV, Joazeiro PP et al (2014) Avian extraintestinal *Escherichia coli* exhibits enterotoxigenic-like activity in the in vivo rabbit ligated ileal loop assay. Foodborne Pathog Dis 11:484–489

Manges AR, Johnson JR (2012) Food-borne origins of *Escherichia coli* causing extraintestinal infections. Food Safety 55:712–719

Marre R, Kreft B, Hacker J (1990) Genetically engineered S and F1C fimbriae differ in their contribution to adherence of *Escherichia coli* to cultured renal tubular cells. Infect Immun 58:3434–3437

Mathers AJ, Peirano G, Pitout JDD (2015) *Escherichia coli* ST131: the quintessential example of an international multiresistant high-risk clone. Adv Appl Microbiol 90:109–154

Maturana VG, de Pace F, Carlos C et al (2011) Subpathotypes of avian pathogenic *Escherichia coli* (APEC) exist as defined by their syndromes and virulence traits. Open Microbiol J 5:55–64

Mellata M, Ameiss K, Mo H et al (2010) Characterization of the contribution to virulence of three large plasmids of avian pathogenic Escherichia coli X7122 (O78:K80:H9). Infect Immun 78:1528–1541

Mills M, Meysick KC, Brien ADO (2000) Cytotoxic necrotizing factor type 1 of uropathogenic *Escherichia coli* kills cultured human uroepithelial 5637 cells by an apoptotic mechanism. Infect Immun 68:5869

Molina-López J, Aparicio-Ozores G, Ribas-Aparicio RM et al (2011) Drug resistance, serotypes, and phylogenetic groups among uropathogenic *Escherichia coli* including O25-ST131 in Mexico City. J Infect Dev Ctries 5:840–849

Moulin-Schouleur M, Répérant M, Laurent S et al (2007) Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J Clin Microbiol 45:3366–3376

Mulvey MA (2002) Adhesion and entry of uropathogenic *Escherichia coli*. Cell Microbiol 4:257–271

Murray AC (2004) Virulence factors predict *Escherichia coli* colonization patterns among human and animal household members. Ann Intern Med 140:848

Nakamura K, Mase M, Tanimura N et al (1997) Swollen head syndrome in broiler chickens in Japan: its pathology, microbiology and biochemistry. Avian Pathol 26:139–154

Ngeleka M, Kwaga JKP, White DG et al (1996) *Escherichia coli* cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect Immun 64:3118–3126

Ngeleka M, Brereton L, Brown G et al (2002) Pathotypes of avian *Escherichia coli* as related to *tsh*, *pap*, *pil* and *iuc* DNA sequences, and antibiotic sensitivity of isolates from internal tissues and the cloacae of broilers. Avian Dis 46:143–152

Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V et al (2008) Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. J Antimicrob Chemother 61:273–281

Nógrády N, Pászti J, Pikó H et al (2006) Class 1 integrons and their conjugal transfer with and without virulence-associated genes in extra-intestinal and intestinal *Escherichia coli* of poultry. Avian Pathol 35:349–356

Nowicki B, Nowicki S (2013) DAF as a therapeutic target for steroid hormones: implications for host-pathogen interactions. Adv Exp Med Biol 735:83–96

Olsen AW, Van Pinxteren LAH, Okkels LM et al (2001) Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. Infect Immun 69:2773–2778

Parker AS, Cerhan JR, Lynch CF et al (2004) History of urinary tract infection and risk of renal cell carcinoma. Am J Epidemiol 159:42–48

Petersen A, Christensen JP, Kuhnert P et al (2006) Vertical transmission of a fluoroquinolone-resistant *Escherichia coli* within an integrated broiler operation. Vet Microbiol 116:120–128
Pichon C, Héchard C, Merle L et al (2009) Uropathogenic *Escherichia coli* AL511 requires flagellum to enter renal collecting duct cells. Cell Microbiol 11:616–628
Pilatti L, de Paiva JB, Rojas TCG et al (2016) The virulence factor *ychO* has a pleiotropic action in an avian pathogenic *Escherichia coli* (APEC) strain. BMC Microbiol 16:35
Poolman JT, Wacker M (2016) Extraintestinal pathogenic *Escherichia coli*, a common human pathogen: challenges for vaccine development and progress in the field. J Infect Dis 213:6–13
Pourbakhsh SA, Boulianne M, Martineau-Doizé B et al (1997) Virulence mechanisms of avian fimbriated *Escherichia coli* in experimentally inoculated chickens. Vet Microbiol 58:195–213
Qabajah M, Awwad E, Ashhab Y (2014) Molecular characterisation of *Escherichia coli* from dead broiler chickens with signs of colibacillosis and ready-to-market chicken meat in the West Bank. Br Poult Sci 55:442–451
Rojas TCG, Parizzi LP, Tiba MR et al (2012) Draft genome of a Brazilian avian-pathogenic *Escherichia coli* strain and in silico characterization of virulence-related genes. J Bacteriol 194:3023
Rojas TCG, Maluta RP, Parizzi P et al (2013) Genome sequences of avian pathogenic *Escherichia coli* strains. Genome Announc 1:2–3
Russo TA, Johnson JR (2003) Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. Microbes Infect 5:449–456
Samuelsson P, Hang L, Irjala H et al (2004) Toll-like receptor 4 expression and cytokine responses in the human urinary tract mucosa. Infect Immun 72:3179–3186
Schouler C, Schaeffer B, Brée A et al (2012) Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. J Clin Microbiol 50:1673–1678
Sharma H, Tal R, Clark N et al (2014) Microbiota and pelvic inflammatory disease. Semin Reprod Med 32:043–049
Skeiky YAW, Coler RN, Brannon M et al (2002) Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL® adjuvant. Vaccine 20:3292–3303
Skyberg JA, Johnson TJ, Johnson JR et al (2006) Acquisition of avian pathogenic *Escherichia coli* plasmids by a commensal *E. coli* isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. Infect Immun 74:6287–6292
Spurbeck RR, Dinh PC, Walk ST et al (2012) *Escherichia coli* isolates that carry *vat*, *fyuA*, *chuA*, and *yfcV* efficiently colonize the urinary tract. Infect Immun 80:4115–4122
Stehling EG, Campos TA, Brocchi M et al (2008) The expression of plasmid mediated afimbrial adhesin genes in an avian septicemic *Escherichia coli* strain. J Vet Sci 9:75
Stenutz R, Weintraub A, Widmalm G (2006) The structures of *Escherichia coli* O-polysaccharide antigens. FEMS Microbiol Rev 30:382–403
Sun D, Wang B, Zhu L et al (2013) Block and boost DNA transfer: opposite roles of OmpA in natural and artificial transformation of *Escherichia coli*. PLoS One 8:59019
Tivendale KA, Noormohammadi AH, Allen JL et al (2009) The conserved portion of the putative virulence region contributes to virulence of avian pathogenic *Escherichia coli*. Microbiology 155:450–460
Totsika M, Moriel DG, Idris A et al (2012) Uropathogenic *Escherichia coli* mediated urinary tract infection. Curr Drug Targets 13:1386–1399
Uehling DT, Hopkins WJ, Elkahwaji JE et al (2003) Phase 2 clinical trial of a vaginal mucosal vaccine for urinary tract infections. J Urol 170:867–869
Ulett GC, Valle J, Beloin C (2007) Functional analysis of antigen 43 in uropathogenic *Escherichia coli* reveals a role in long-term persistence in the urinary tract. Infect Immun 75:3233–3244
Verma R, Rojas TCG, Maluta RP et al (2015) Fimbria-encoding gene *yadC* has a pleiotropic effect on several biological characteristics and plays a role on avian pathogenic *Escherichia coli* (APEC) pathogenicity. Infect Immun 84:187–193
Vincent C, Boerlin P, Daignault D et al (2010) Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg Infect Dis 16:88–95

Wolfs TGAM, Buurman WA, van Schadewijk A et al (2002) In vivo expression of toll-like receptor 2 and 4 by renal epithelial cells: IFN- and TNF-mediated up-regulation during inflammation. J Immunol 168:1286–1293

Yang H, Chen S, White DG et al (2004) Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolates from diseased chickens and swine in China. J Clin Microbiol 42:3483–3489

Chen Y-H, Hsueh PR (2012) Changing bacteriology of abdominal and surgical sepsis. Curr Opin Infect Dis 25:590–595

Zalewska-Piatek BM (2011) Urinary tract infections of *Escherichia coli* strains of chaperone-usher system. Pol J Microbiol 60:279–285

Zanella A, Alborali GL, Bardotti M et al (2000) Severe *Escherichia coli* O111 septicaemia and polyserositis in hens at the start of lay. Avian Pathol 29:311–317

Zhao S, Maurer JJ, Hubert S et al (2005) Antimicrobial susceptibility and molecular characterization of avian pathogenic *Escherichia coli* isolates. Vet Microbiol 107:215–224

Chapter 10
Secretion Systems of Pathogenic *Escherichia coli*

Fernando Navarro-Garcia, Fernando Ruiz-Perez, Mariano Larzábal, and Angel Cataldi

Summary Protein secretion plays a central role in modulating the interactions of bacteria with their environments. Bacterial ribosomes synthesize up to 8000 different proteins. Almost half of these become integrated in membranes and are secreted to the periplasm or to the external milieu. Many bacterial processes, such as DNA replication, motility, transport, antibiotic resistance, scavenging of chemicals, and pathogenesis, depend on protein secretion. Thereby, evolutionarily unrelated protein nanomachines have been developed, which allow exported proteins to cross the Gram-negative membranes. Bacterial proteins can be exported directly from the cytoplasm out of the cell by a one-step (cytoplasm to extracellular milieu), including the type I secretion system (T1SS), T3SS, T4SS, and T6SS, or two-step (periplasm translocation step), including the T2SS and T5SS, while the T4SS can use either the one- or two-step mechanism. The T3SS, T5SS, and T6SS are the more common secretion systems in *Escherichia coli* and most of the secreted substrates are virulence factors related to pathogenic *E. coli*. In this chapter, we will describe the main characteristic of these last three secretion systems.

F. Navarro-Garcia (✉)
Department of Cell Biology, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), México DF, Mexico
e-mail: fnavarro@cell.cinvestav.mx

F. Ruiz-Perez
Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, VA, USA
e-mail: FR3D@hscmail.mcc.virginia.edu

M. Larzábal • A. Cataldi
Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina
e-mail: larzabal.mariano@inta.gob.ar; cataldi.angeladrian@inta.gob.ar

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_10

1 Introduction

The structures and composition of inner membrane (IM) and outer membrane (OM) of Gram-negative envelope are different. The IM is a classical phospholipid bilayer and IM proteins often are integral proteins crossing the membrane with one or more hydrophobic α-helices. A few lipoproteins are also anchored to the outer leaflet of the IM via a lipid moiety. In contrast, the OM is an asymmetric bilayer composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) and outer leaflet (Ruiz et al. 2006). The protein in the OM can be lipoproteins or integral membrane proteins; most of the lipoproteins are anchored by a lipid moiety in the inner leaflet of the OM and face the periplasm, while integral membrane proteins are known as OMPs. OMPs serve as channels or porins because they adopt a β-barrel conformation, which allows free diffusion of ions and hydrophilic molecules across the membrane. The IM and the OM are separated by the periplasm, which comprises about 10 % of the cell volume and is a highly viscous compartment that is occupied by soluble proteins and a thin layer of peptidoglycan. The periplasm contains more than 300 proteins (Weiner and Li 2008), which perform a large variety of physiological functions, such as protein folding, uptake and transport of nutrients, and detoxification of harmful substances.

The majority of secreted proteins carry a signal sequence recognized by the Sec apparatus, which transports them through the IM in an unfolded conformation. In this context, proteins can be exported directly from the cytoplasm out of the cell by a one-step or two-step process. In one-step mechanism, the substrates are transported directly from the bacterial cytoplasm into the extracellular space or into a target cell and include the type 1 secretion system (T1SS), T3SS, T4SS, and T6SS. Whereas, in two-step secretion mechanism the substrates are first translocated into the periplasmic space by IM-spanning transporters such as the SecYEG translocon or the Tat system (twin-arginine translocation) and are subsequently transferred to the OM or secreted into the extracellular space by a dedicated OM-spanning secretion system and include the T2SS and T5SS. The T4SS can use either the one- or two-step mechanism (Costa et al. 2015).

An example of the two-step process is the T2SS, which is found in a wide variety of Gram-negative bacteria. The T2SS comprises four parts: an OM complex, a periplasmic pseudopilus, an IM platform, and a cytoplasmic ATPase. The OM complex is formed by the dodecameric protein GspD, the T2SS secretin (Reichow et al. 2010). The system secretes folded proteins from the periplasm into the extracellular environment. Virulence determinants secreted by T2SS include the ADP-ribosylating toxins of enterotoxigenic *E. coli* (heat labile toxin), *Vibrio cholerae* (cholera toxin), and *Pseudomonas aeruginosa* (exotoxin A). The one-step process of the T1SS compresses three major components: ATP-binding cassette (ABC) transporters, outer membrane factors (OMFs), and membrane fusion proteins (MFP). The secreted substrate in uropathogenic strains of *E. coli* is the hemolytic toxin called HlyA (Uhlen et al. 2000). In this chapter, we will extend the information about the secretion systems by further developing the T3SS, T5SS, and T6SS, which are common in pathogenic *E. coli* and most of the secreted substrates are virulence factors and linked to pathogenesis in mammals.

2 The Type 3 Secretion System (T3SS)

T3SS is a protein export pathway that is formed by an assembled complex apparatus from more than 20 components (Fig. 10.1). At its core lie the injectisome and a complex network of specialized chaperones that target secretory proteins to the antechamber of the injectisome (Portaliou et al. 2016). This machinery is used to assemble the needle complex of many Gram-negative pathogens, which injects effector proteins into host cells and elicit changes associated with disease. The T3SS creates a contiguous channel through the bacterial and host membranes, allowing injection of specialized bacterial effector proteins directly to the host cell. The delivery of effector proteins into a host cell via T3SS allows the bacterium to manipulate the bacterial-host cell interaction in its favor by targeting a wide variety of cellular processes including the cytoskeleton, phagocytosis, apoptosis, and the inflammatory response (Navarro-Garcia et al. 2013; Sanchez-Villamil and Navarro-Garcia 2015). The T3SS is widespread in many Gram-negative bacteria, including

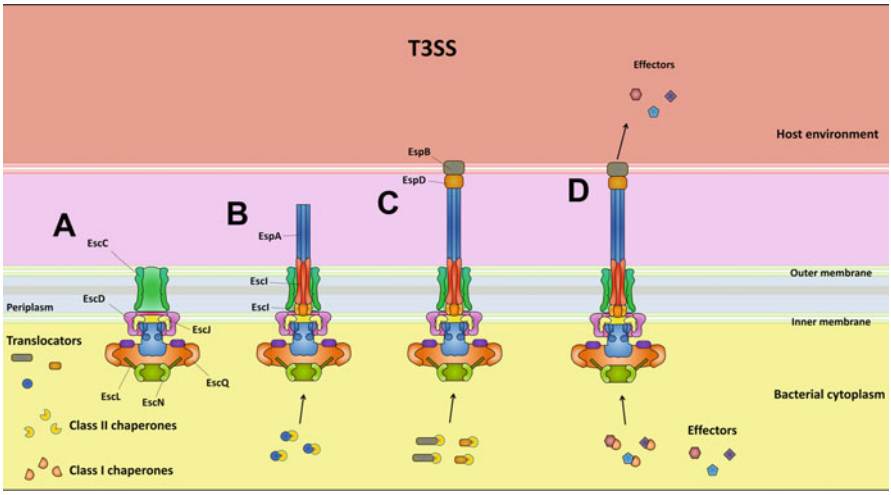

Fig. 10.1 T3S hierarchy and timing. Assembly of the type III secretion holocomplex starts from two membranes: The secretin ring (EscC) in the OM assembles independently and scaffolds assembly of the outer MS ring (EscD). (*A*) In the inner membrane, assembly nucleates at the minor export apparatus proteins (EscRST) and progresses via the recruitment of the switch protein and major export apparatus protein (EscUV). Both the secretin-outer MS ring protein complex and the export apparatus complex can associate with the inner MS ring protein (EscJ). Integration of the two assemblies into one complex allows subsequent recruitment of the cytoplasmic components. (*B*) Secretion of early substrates (EscF, EscI, and EscP) leads to assembly of the needle filament until it reaches a specific length. The needle length is controlled by the needle length regulator (EscP), which switches substrate specificity to the secretion of intermediate substrates (needle tip protein: EspA), presumably through interaction with the switch protein. (*C*) The needle tip complex senses contact with the host cell membrane. The contact signal is transduced through the needle filament down to the cytoplasmic side of the base, where it leads to dislodging of the gatekeeper. (*D*) This frees the way for secretion of late substrates (translocon and effector proteins) and their subsequent injection into the host cell cytoplasm

symbionts, such as *Rhizobium*, plant pathogens as *Pseudomonas syringe* and human pathogens such as plague (*Yersinia pestis*), typhoid fever (*Salmonella enterica* serovar Typhi), gastroenteritis (*Shigella flexneri*), and infantile bacterial diarrhea (enteropathogenic and enterohemorrhagic *E. coli*; EPEC and EHEC).

2.1 Injectisome

The first electron micrographs of *Salmonella* (Kubori et al. 1998) and *Shigella* (Blocker et al. 2001) T3SS unveiled a remarkable nanosyringe-like structure, characterized by a wide base embedded in the membrane of the bacteria and a thin extracellular needle that protruded to the extracellular space (Fig. 10.1). The architecture of the T3SS is conserved in different bacterial species, as are the functions of its main components. Assembly can be categorized into four discrete stages: (1) assembly of the basal body rings and export apparatus, (2) assembly of the inner rod and needle, (3) assembly of the tip and translocon, and (4) secretion of effectors. Nanosyringe-like structures share high structural and genetic homology with the flagellar apparatus and with parts of the F1F0 ATP synthases, which suggest a common ancestry (Kawamoto et al. 2013). Due to disparate assignment of protein and gene names, common nomenclature rules have been proposed for reducing confusion (Hueck 1998) (Table 10.1), including the secretion and cellular translocation (Sct) moniker for the highly conserved genes.

The injectisome is formed by three parts: the extracellular segment, the basal body, and the peripheral IM cytoplasmic components. The extracellular segment pipes the bacterial OM to the host plasma membrane and it comprises the needle connecting either a tip or a filament and the translocator pore. The basal body, which crosses both membranes of the Gram-negative envelope, is built of stacked toroids: an OM ring, which extends to the periplasm and associates with the IM ring. Thus, the basal body and the extracellular segment form a conduit through which effectors are transferred. The cytoplasmic components are the ATPase complex and the cytoplasmic ring that is known as C-ring; these two components are essential for protein secretion, protein sorting, and the unfolding of secretory proteins (Portaliou et al. 2016).

The injectisome assembly requires the Sec machinery and then the T3SS itself. After the basal body is made, by using the T3SS, the injectisome is completed with an internal connector of the basal body toroids, elongation of the needle, and the formation of the translocator pore. The membrane-embedded ring-like structures, compressing the basal body, connect the IM and OM by forming a continuous tubular conduit (Radics et al. 2014).

The needle or filament length varies between different bacteria and is dictated by the molecular ruler SctP (EscP) (Monjaras Feria et al. 2012). Inside, the base of the needle is associated with the inner membrane platform by a “socket”, whose lower part, termed the “cup”, is oriented toward the cytoplasm. It is conceivable that the unfolded effectors are delivered into the cup, after which they are exported through the hollow cavity of the needle (Hayes et al. 2010).

Table 10.1 Unified nomenclature, localization, and function of T3S injectisomes and flagellum

Common name	Function	EPEC/EHEC	*S. flexneri*	*Yersinia*	Flagellar apparatus
Intracellular components					
SctF	Needle	EscF	MxiH	YscF	–
SctA	Tip/filament	EspA	IpaD	LcrV	FliC
SctB	Translocator	EspB	IpaC	YopD	–
SctE	Translocator	EspD	IpaB	YopB	–
Basal body					
SctC	OM ring	EscC	MxiD	YscC	FlgI, FlgH
–	Pilotin	–	MxiM	YscW	–
SctI	Inner rod	EscI	MxiI	YscI	FlgB/C/F/G
SctD	IM ring	EscD	MxiG	YscD	FliG
SctJ	IM ring	EscJ	MxiJ	YscJ	FliF
SctR	Export apparatus	EscR	Spa24 (SpaP)	YscR	FliP
SctS	Export apparatus	EscS	Spa9 (SpaQ)	YscS	FliQ
SctT	Export apparatus	EscT	Spa29 (SpaR)	YscT	FliR
SctU	Export apparatus	EscU	Spa40 (SpaS)	YscU	FlhB
SctV	Export apparatus	EscV	MxiA	YscV	FlhA
Cytoplasmic components					
SctQ	Cytoplasmic ring	SepQ	Spa33	YscQ	FliM, FliN
SctL	Stator	EscL	MxiN	YscL	FliH
SctN	ATPase	EscN	Spa47	YscN	FliI
SctO	Stalk	EscO	Spa13	YscO	FliJ
SctP	Molecular ruler	EscP	Spa32	YscP	FliK
SctW	Gate-keeper	SepL	MxiC	YscN/TyeA	–
–	Regulatory comp.	SepD	–	–	–
SctK	ATPase co-factor	–	MxiK	YscK	–
Chaperones					
–	Early substrates	EscE, EscG		YscE, YscG	
–	Middle substrate	CesAB/D/D2	IpgC, OpgD	LcrG, SycD/B	FliS, FliT
–	Late substrate	CesT, CesF	IpgE/A, Spa1	SycE/T/H/N, YcscB, YsaK	

Note: EPEC/EHEC and *S. flexneri* (EIEC) are compared with prototypical *Yersinia* injectisome

2.2 T3SS Translocators: EspA, EspB, and EspD

EspA, EspB, and EspD are proteins encoded by *LEE4* operon that make up the translocon portion (Fig. 10.1) of the T3SS in EHEC and EPEC (Croxen et al. 2013; Navarro-Garcia et al. 2013; Sanchez-Villamil and Navarro-Garcia 2015). EspA makes hollow, filamentous appendages surrounding the bacteria, which are present in a transient manner. These structures form a translocation tube that acts as a channel

to deliver proteins from the bacteria into the intestinal cell. EspB and EspD are involved in pore formation on the membranes of the infected cells (Ide et al. 2001) and are translocated to both the membrane and the cytoplasm. Complexes formed by EspA, EspB, and EspD proteins may participate in the initial step of bacterial adherence (Nougayrede et al. 2003).

The proximal end of the EspA filament rests in the EscF needle near the basal body of the T3SS. EspA caps and nucleates around EscF, also encoded in *LEE4*. The assembly of EspA subunits is guided by coiled-coil regions. EspA filaments are elongated by addition of EspA subunits to the tip of the growing filament and the filament length is modulated by the availability of intracellular EspA subunits (Crepin et al. 2005).

EspB and EspD represent the tip of the needle forming a pore into target cell membranes. The pores are composed of 6–8 subunits. EspB and EspD can associate with erythrocyte membranes and both proteins with each other in vitro. The homologies of EspB and EspD to functional domains of other pore-forming proteins (Yop/Ipa) support the idea that both proteins are involved in pore formation. The N-terminal segment of EspD, encompassing residues 1-171, containing two amphipathic domains is critical for EspD oligomerization. In turn, EspD interacts with EspB and EspA. Two regions on either side of a putative transmembrane domain of EspB are required for the binding of EspB to EspD (Luo and Donnenberg 2011).

SepD and SepL are essential for translocator secretion; their lack leads to hypersecretion of effectors and arresting of translocator secretion. The translocators need chaperones to be secreted and translocated by the T3SS. CesD is a cytoplasmic chaperone required for proper EspB and EspD secretion. CesD2 is a second LEE-encoded chaperone for EspD (Neves et al. 2003). Additionally, CesAB is a chaperone for EspA and EspB (Creasey et al. 2003).

2.3 T3SS Effectors

T3SS-producing bacteria utilize their nanosyringe-like T3SS to translocate effector proteins into host cells (Fig. 10.1), which facilitate bacterial colonization, survival, and immune evasion in infected hosts (Croxen et al. 2013; Navarro-Garcia et al. 2013; Sanchez-Villamil and Navarro-Garcia 2015). The translocated T3SS effector proteins from EHEC, EPEC, and enteroinvasive *E. coli* (EIEC) (which is genetically and biochemical equivalent to *Shigella*) affect diverse signaling pathways and physiological processes. In EPEC and EHEC, seven basic effectors are encoded with the T3SS translocation machinery on the chromosomal pathogenicity island, LEE, while other effectors are encoded within prophages and other integrative elements, which are called non-LEE effectors. Although EPEC and EHEC show high levels of conservation between the LEE-encoded effectors, their non-LEE effector repertoire is significantly diverse. EPEC strains E2348/69 and B171 encode at least 23 intact effector genes (Deng et al. 2012), whereas the EHEC O157 Sakai about 50 T3SS effectors. Unlike the chromosomal LEE T3SS, the *Shigella* T3SS is encoded

on a large virulence plasmid along with the majority of identified T3SS effectors. About 30 T3SS effectors are currently recognized in *Shigella* (Parsot 2009).

2.3.1 Re-programming Cytoskeleton

EPEC, EHEC, and *Shigella* have acquired a subset of effectors to promote the colonization of their host by modulating the host cytoskeleton (Navarro-Garcia et al. 2013). Effectors secreted by these bacteria accomplish diverse molecular mechanisms for pedestal formation, cell invasion, modulation of Rho GTPases, and reorganization of microtubules and intermediate filaments.

EPEC and EHEC colonize the human intestinal mucosa, form characteristic histological lesions (attaching and effacing, A/E) on the infected epithelium, and T3SS is required. The T3SS effector Tir not only acts as the host cell receptor for intimin, but, upon binding, intimin initiates Tir clustering which mediates downstream signaling events leading to the formation of actin-rich pedestals or A/E lesion (Campellone et al. 2004).

In EPEC, phosphorylation of Tir in the tyrosine residue 474 (Y474) by alternative host cell tyrosine kinases c-Fyn recruits the host cell adaptor protein Nck. Nck then activates the neural Wiskott-Aldrich syndrome protein (N-WASP) to stimulate Arp2/3-mediated actin polymerization by binding to the WASP-homology 1 (WH1) domain of N-WASP (Lommel et al. 2001). It has been proposed that Tir:Nck pathway recruits N-WASP via both WIP/WH1 and Nck/WH1 interactions to synergistically trigger actin assembly (Weisswange et al. 2009).

EHEC Tir lacks a Y474 equivalent and the actin polymerization process is mediated via the non-LEE-encoded T3SS translocated effector protein, TccP (Garmendia et al. 2004), also known as EspFU (Campellone et al. 2004). Rather than mimicking Cdc42 activation, each TccP/EspFU proline-rich repeat mimics an auto-inhibitory element within the GBD, thus competitively binding to the GBD to relieve N-WASP autoinhibition, triggering actin polymerization via the Arp2/3 complex (Cheng et al. 2008).

The uptake of *Shigella*/EIEC by the host cell requires a complex rearrangement of the host cell membrane and cytoskeleton, which is initially triggered by receptor binding, but depends on the T3SS effectors for complete uptake. Bacteria can also be captured by filopodial extensions from the host cell, termed nanometer-thin micropodial extensions (NMEs), and this interaction occurs through the T3SS tip complex proteins IpaB and IpaD (Romero et al. 2011). For the invasion, Erk1/2 is activated by connexin-mediated signaling and extracellular ATP, which controls actin retrograde flow in NMEs resulting in NME retraction, getting the bacterium in contact with the cell membrane for invasion. Bacterial invasion initiates with the formation of membrane ruffles, which are modulated by two effectors associated with Rho GTPase activity, IpgB1 and 2. The C-terminal domain of IpaC induces actin polymerization, which leads to the formation of cell extensions that engulf the bacterium.

The Rho family GTPases are crucial in the regulation of key cellular functions and the best characterized members are Cdc42, Rac1, and RhoA, which trigger filopodia, lamelipodia/ruffles, and stress fibers, respectively. A number of bacterial

effector proteins modulate Rho GTPases and many were grouped on the basis of a WxxxE motif (Alto et al. 2006). Representatives of this family of effectors act as RhoGEFs by binding to their respective Rho GTPases and inducing a conformational change to allow GTP binding and hence activation. These effectors include the EPEC/EHEC effectors Map, EspM, and EspT, and the *Shigella* effectors IpgB1 and IpgB2. Map and EspM exhibit guanine exchange factor (GEF) activity towards Cdc42 and RhoA (Arbeloa et al. 2010), respectively, thereby triggering formation of filopodia (Map) and stress fibers (EspM) (Bulgin et al. 2009). EspT activates both Cdc42 and Rac1, which trigger membrane ruffles, lamellipodia, and EPEC-induced invasion (Bulgin et al. 2009). Whereas, *Shigella* IpgB1 exhibits GEF activity towards both Cdc42 and Rac1, which trigger membrane ruffles and lamellipodia, and IpgB2 activates RhoA, triggering formation of stress fibers (Huang et al. 2009).

Regarding the microtubules, the *Shigella* T3SS effector VirA is required for the entry of bacteria into epithelial cells. Interestingly, EspG effector from EPEC and EHEC is a VirA homologue and also induces microtubule disruption, resulting in actin stress fiber formation by releasing GEF-H1 from the cytoskeleton and by activating RhoA (Matsuzawa et al. 2004). Regarding to intermediate filaments, it was found that CK8 and CK18 are recruited to the site of bacterial attachment. Tir interacts with both CK18 (Batchelor et al. 2004) and an adaptor protein, the 14-3-3 tau isoform, which also binds CK18. Formation of the Tir:CK18:14-3-3 tau complex is essential for biological activity, as depletion of either CK18 or 14-3-3 tau impaired pedestal formation (Batchelor et al. 2004). The CK18 and the 14-3-3 zeta isoform also bind EspF (Viswanathan et al. 2004), allowing Tir and EspF to coordinateProtein secretion systems: collapse of the intermediate filament network with actin redistribution and polymerization. Thus, EPEC/EHEC infection could cause cytoskeletal restructuring at the site of bacterial attachment where actin and intermediate filament components accumulate while microtubule components are depleted.

2.3.2 Manipulating the Immune Responses

The host immune system is the main obstacle in bacterial infections and its manipulation is essential for successful bacterial colonization and dissemination. EPEC/EHEC modulate the innate immunity, including evasion of phagocytosis and autophagy in order to survive and proliferate (Sanchez-Villamil and Navarro-Garcia 2015).

Phagocytosis is a key mechanism utilized by the innate immune system to fight invading pathogens. EPEC is able not only to block its own uptake by professional phagocytes (*cis*-phagocytosis), but is also able to inhibit the phagocytosis of IgG-opsonized particles (*trans*-phagocytosis) (Quitard et al. 2006). Inhibition of both *cis*- and *trans*-phagocytosis by EPEC/EHEC is T3SS-dependent and four effectors have been characterized as inhibitors of phagocytosis: EspF, EspB, EspJ, and EspH.

The nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPK) are pro-inflammatory signaling pathways, which regulate the production and secretion of inflammatory mediators. EPEC/EHEC effectors antagonize the MAPK and NF-kB signaling pathways and inhibit the production of inflammatory mediators

throughout the course of infection. EPEC initially activates the NF-kB signaling pathway through a T3SS-independent mechanism, and subsequently utilizes a T3SS-dependent mechanism to inhibit NF-kB activation and production of pro-inflammatory cytokines (Maresca et al. 2005). It has been demonstrated that EPEC and EHEC encode a set of effector proteins (NleE, NleB, NleC, NleD, and NleH) that specifically target NF-kB complex components, while other effectors target NF-kB signaling (NleE, NleB, Tir, and EspT).

During invasion and multiplication within the cells of the intestinal epithelium, *Shigella*/EIEC peptidoglycan stimulates the intracellular receptor NOD1, which interacts with the serine-threonine kinase RICK to induce NF-kB and MAPK p38, ERK, and JNK signaling. *Shigella* T3SS-delivered effectors target components of both MAPK and NF-kB pathways (Ashida et al. 2011), involving OspZ and OspG (homologs of NleE and NleH). IpaH9.8 translocates to the nucleus and acts as an E3 Ub ligase. Finally, OspI selectively deamidates a glutamine residue to glutamic acid in the E2 enzyme UBC13. Additionally, *Shigella* through OspI activity blocks NF-kB-mediated inflammatory response at an early stage of epithelial invasion (Sanada et al. 2012).

2.3.3 Regulating the Cell Survival

EPEC, EHEC, and *Shigella* have developed a large panel of effectors able to modulate the cell death program by regulating cell survival and apoptosis. Apoptosis is a programmed cell death that can proceed through two pathways: extrinsically, receptor-mediated pathway, and intrinsically, mitochondria-mediated pathway (Rudel et al. 2010). Both pathways lead to the activation of caspases, which irreversibly cleave essential cellular proteins, committing cells to death.

EspF, Map, and Cif are a subset of effectors that promote cell death in EPEC and EHEC. EspF and Map contain a canonical N-terminal mitochondrial targeting sequence for its importation into the mitochondrial matrix by the host machinery (Ma et al. 2006). EspF and Map destabilize mitochondrial membrane potential, leading to cytochrome c release, caspase activation, and downstream intrinsic apoptosis (Ma et al. 2006). Cycle inhibiting factor (Cif) was found to halt cell cycle progression, and following a prolonged infection and cell cycle arrest, Cif induces delayed apoptosis (Samba-Louaka et al. 2009).

The extrinsic pathway of apoptosis is activated upon recognition of extracellular danger signals by cell surface death receptors. TNFR1-associated death domain protein (TRADD) and Fas-associated DD protein (FADD) function in downstream signaling complexes that activate caspase-8, which proteolytically activates downstream executioner caspases, promoting apoptosis (Strasser et al. 2009). NleD-mediated cleavage of JNK also inhibits extrinsic apoptosis in infected cells. NleB1 and NleB2 participate in survival signaling during EPEC/EHEC infection (Pearson et al. 2013). NleB1 GlcNAcylates conserved arginine residues in an irregular way (instead serine/threonine) within the DDs of adaptor proteins FADD, TRADD, and RIPK1 (Pearson et al. 2013).

The intrinsic pathway is triggered by a variety of intracellular stresses and results in the permeabilization of the outer mitochondrial membrane and subsequent release of cytochrome c into the cytosol. NleH1 interacts with Bax inhibitor-1 (BI-1) in a kinase-independent manner, promoting BI-1 anti-apoptotic inhibition of Bax (Robinson et al. 2010). EspH contributes to caspase-3 activation and the induction of intrinsic apoptosis. Interestingly, EspT and EspM2 are bacterial RhoGEF mimics that are unaffected by EspH inhibition and act to inhibit EspH-induced intrinsic apoptosis. EspZ inhibits intrinsic apoptosis and promotes host survival that ultimately results in the inhibition of caspase activation; and EspZ activity counters the cytotoxic effects of Map and EspF within mitochondria. Finally, NleF, acting as a general apoptosis inhibitor, directly inhibits caspases involved in both intrinsic and extrinsic apoptotic pathways, including caspases-4, -8, and -9 (Blasche et al. 2013).

Autophagy is a conserved catabolic pathway that allows eukaryotes to degrade proteins and organelles by sequestering them in specialized double-membrane vesicles named autophagosomes. VirG/IcsA, which is required for actin-based motility for bacterial spreading, is targeted by the autophagic component Atg5. Strikingly, *Shigella* can evade autophagy by delivering IcsB, which competitively binds to IcsA to block Atg5 binding and enables the bacteria to evade autophagic recognition (Ogawa et al. 2005).

2.4 T3SS Regulators

Regulation of LEE gene expression is a highly complex and coordinated process, dependent on environmental conditions, quorum sensing, and several regulators and regulatory pathways, in a direct or indirect way. T3SS is not constitutively expressed, but it is synthesized in response to environmental signals characteristic of mammalian internal media (e.g. DMEM media) (Rosenshine et al. 1996). T3SS does not appear in bacterial media as LB or M9 and it needs pH 7.4–7.5, bicarbonate, Ca^{2+}, and Fe^{3+}.

The central regulator that controls the expression of LEE genes is Ler, encoded by *LEE1* operon. Ler activates all *LEE* operons, except *LEE1*, highlighting the crucial role of Ler in the regulation of all genes important for A/E lesion formation. The Ler regulon extends beyond LEE and includes unlinked pathogenicity genes; more than 50 genes in EPEC E2348/69, among them *map*, *espG,* and *escD*, and four extra-LEE genes in EHEC EDL933 (Bingle et al. 2014). Global regulator of LEE activator (GrlA) and global regulator of LEE repressor (GlrR) are also LEE-encoded regulators. GrlR-GrlA regulatory system also controls flagellar and hemolysin *ehxCABD* operon expression in EHEC (Iyoda et al. 2006). EPEC PerC induces *ler* transcription independently of GrlA (Bustamante et al. 2011). EHEC lacks pEAF plasmid, but there is a family of functional prophage-encoded PerC homologous proteins, called PchA, PchB, and PchC, which play an important role in *ler* expression in EHEC (Iyoda and Watanabe 2005).

Other regulators play an important role in the repression of *LEE* operons, such as the nucleoid-associated proteins, H-NS (Deng et al. 2004), GrlR independently of its interaction with GrlA (Jimenez et al. 2010), Hha, and Hfq (destabilizing *grlRA* mRNA). On the other hand, regulators that positively regulate *LEE* operons include: integration host factor (IHF), Ler that can neutralize the silencing exerted by H-NS on LEE promoters (Bustamante et al. 2011), Fis, sigma factor S (σS) (Mitra et al. 2012), and sigma N (σN) in EHEC O157:H7. Non-coding RNAs (ncRNAs) have recently emerged as important regulators involved in controlling the expression of different genes. DsrA enhances *ler* expression by improving the levels of functional transcripts. GlmY and GlmZ also play global roles in gene expression that included post-transcriptional degradation of *LEE4* and *LEE5* polycistronic mRNA, stimulating the translation of the non-LEE-encoded effectors NleA and EspFu providing further evidence of the complexity of LEE control (Gruber and Sperandio 2015).

Environmental and nutritional factors play a role in the regulation of LEE: short-chain fatty acids inhibit the growth of EHEC, but at low concentrations enhance the expression of the virulence genes; FusKR senses fucose and represses the expression of LEE genes; Ethanolamine (EA) positively regulates *ler* expression in EHEC (Kendall et al. 2012). Expression of *ler* is directly inhibited by glycolytic byproducts and promoted by gluconeogenic conditions respectively by the catabolite/osmotic stress-responsive Cra/KdpE system, which converge in the regulation of the LEE T3SS and several non-LEE-encoded effectors (Njoroge et al. 2013).

Intra and interspecies communication through quorum sensing (QS) systems comprises bacterial sensing of own chemical signals and host adrenergic hormones. Three systems are described in Gram-negative bacteria: (1) AI-1 or LuxR/I System, (2) AI-2 or LuxS System, (3) AI-3/epinephrine/norepinephrine system. *E. coli* may possess the whole or partial components of the three systems (Yang et al. 2014). Mechanisms of LuxR/I and AI-3/epinephrine/norepinephrine systems were shown to regulate LEE gene expression in EPEC and EHEC strains. The role of the AI-2 or LuxS system is less understood in these strains.

3 The Type 5 Secretion System (T5SS)

The type 5 secretion pathway is the most common mechanism used to deliver surface-exposed or secreted virulence factors by Gram-negative bacteria (Henderson et al. 2004). Members of this system are termed autotransporters (AT) to imply self-sufficiency in their secretion mechanism, now known to be more complex than initially believed given the requirement of other accessory proteins for their secretion.

Three structural and functional domains can be distinguished from a typical AT molecule: the signal peptide; the N-terminal passenger domain (also known as "the α-domain"), which conveys biological functions to the AT-molecule; and the pore-forming C-terminal translocator domain (also designated "β-domain"), which targets the protein to the outer membrane (OM) (Fig. 10.2).

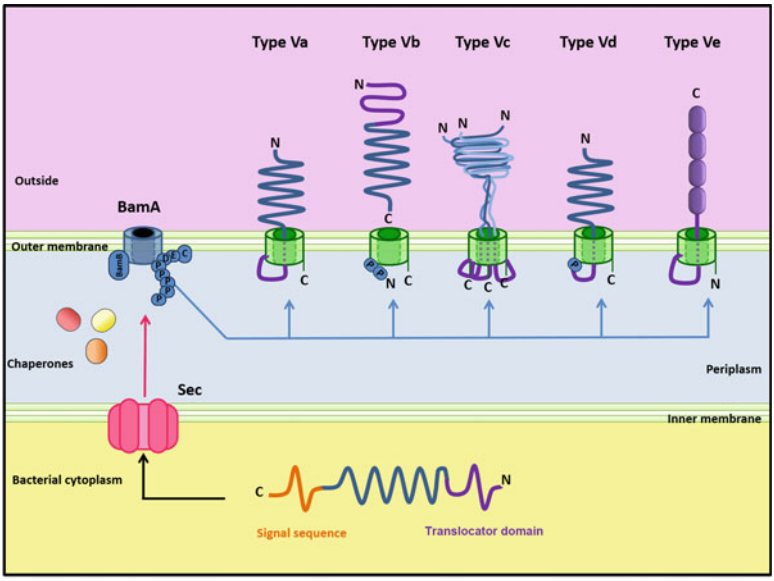

Fig. 10.2 Five subcategories of Autotransporter proteins. The unfolded autotransporter of the T5SS is transferred to the periplasm by the SecYEG translocon, where several chaperones stabilize its unfolded structure. In the next stage, the translocator domain is inserted into the OM with the assistance of BamA, where it folds into a β-barrel. The unfolded passenger domain passes through the pore created by the translocator domain and folds into a β-helical structure. Structures and topology of the different type V secretion systems. The translocation domain is displayed in green, linker/Tps regions in *purple*, and passenger domains in *blue*. POTRA domains are labelled (P). The Type Va autotransporter model. The C-terminal membrane anchor (*green*) is recognized by the POTRA (P) domains of BamA; the Bam complex aids in inserting the β-barrel membrane anchor into the outer membrane (OM). The linker region (*purple*) then forms a hairpin inside the pore of the barrel. The passenger domain (*blue*) is pulled through the pore. The energy for this presumably derives from the sequential folding of the passenger domain on the outside of the cell. Once the passenger domain has been secreted, the linker assumes an α-helical conformation and plugs the pore. Type Vb autotransporter model. Vb subcategory are members of the two-partner secretion system (TPS) in which the passenger and the translocator domain harboring polypeptide-transport-associated (POTRA) motifs. The translocation proteins are known as TpsA and TpsB (*green*), and most of them are encoded in an operon. After their translocation through the inner membrane, TpsB forms the translocation β-barrel, which mediates secretion of the passenger protein (TpsA) through the outer membrane. Type Vc autotransporter model. Characterized by the presence of three passenger domains fused to the one-third segment of a fully functional C-terminal translocator domain allowing secretion of trimeric polypeptides. The Bam complex is required for trimeric autotransporter biogenesis and recognizes the C-terminal membrane anchor (*purple*). The Bam complex assists in trimerization of the β-barrel and membrane insertion. The linker regions (*blue*) form hairpins within the pore, and this leads to translocation of the polypeptides encoding the passenger domain. Type Vd autotransporter model. Autotransporters in this subcategory resemble a fusion between the Va, monomeric ATs, and the Vb, two partner system autotransporters. The passenger domain of PlpD (lipolytic enzyme) is connected to the translocator β-barrel with a POTRA domain and then is released by an autocatalytic reaction after translocation. Type Ve autotransporter model. Ve has an inverted topology with the passenger domain located in the C-terminal and the translocation unit in the N-terminal of the molecule; hence the term inverse autotransporter. Likewise, classical ATs, Ve are transported into the periplasm by the Sec translocon, assisted through the periplasm by common chaperones like SurA, and lastly, inserted into the outer membrane via the BAM complex

3.1 Autotransporter Biogenesis

In the last 5 years, considerable progress has been made to understand the AT biogenesis. However, the precise molecular events on later stages of autotransporter translocation remain unsolved. The current model for AT biogenesis has been proposed based on the classical monomeric autotransporters (Ruiz-Perez and Nataro 2014) (Fig. 10.2). ATs are synthesized in the cytoplasm and then directed to the inner-membrane SecYEG translocon, which catalyzes energy-driven export of ATs into the periplasm, and which may occur by co-translational or post-translational mechanisms. Some ATs suffer post-translational modifications during their trafficking, such as in the case of AT adhesins, which are glycosylated in the cytoplasm by heptosyltransferases before export to the periplasm (Klemm et al. 2006), while other ATs like the serine proteases SphB1 and NalP are lipidated in the periplasm by acyltransferases (Henderson et al. 2004). Once in the periplasm, the AT proteins are protected and preserved in a "translocation-competent" state by common periplasmic chaperones, such as Skp, SurA, and DegP toward the β-barrel assembly machinery (Bam) complex, which assists folding and insertion of OMPs into the OM. Several other more specialized chaperones have been reported to interact with, or to influence, AT secretion, including FkpA, DsbB, and Virk (Ruiz-Perez et al. 2010; Tapia-Pastrana et al. 2012). Once in the periplasm, chaperone-bound ATs are targeted to the Bam complex, in which BamA and BamD and possibly other Bam molecules assist insertion of the AT translocator domain into the OM (Oberhettinger et al. 2015). Interestingly, it was found that ATs could be fully assembled in the yeast mitochondrial OM, where it exists in a native conformation despite the absence of the BAM machinery (Ulrich et al. 2014). Nevertheless, a recent study has shown that the Bam complex and the molecular chaperone SurA are both necessary to promote complete assembly of the EspP autotransporter into proteoliposomes employing purified components (Roman-Hernandez et al. 2014).

Once the AT molecule is positioned into the OM, translocation of the AT passenger domain across its own channel occurs through a mechanism known as the "hairpin model", which entails passage of the polypeptide through the pore beginning with a contortion of the C-terminus of the passenger domain inside the hydrophobic cavity of the translocator domain, and followed by its unfolded or partially folded distant N-terminus towards the external milieu. Evidence of this translocation mechanism has been shown for classic monomeric ATs, the two-partner system (TPS), trimeric, and inverse ATs (Oberhettinger et al. 2015). Recent studies have shown that conserved paired glycine-aromatic "mortise and tenon" motifs in the translocator domain of ATs are essential for ATβ-barrel folding and passenger translocation (Leyton et al. 2014). Similarly, motifs localized in the passenger domain such as the "passenger associated transport repeat" (PATR) motif are necessary for efficient export of the passenger (Doyle et al. 2015). The energy driving translocation of AT across the bacterial OM seems to derive from multiple sources, including the net electrostatic charge and folding properties of the N- and C-terminal regions of the AT passenger domain (Drobnak et al. 2015).

Once on the bacterial surface, the passenger domain of ATs may remain attached to the translocator domain or subsequently released by an autocatalytic mechanism mediated by the serine protease motif in the passenger domain or by a catalytic dyad inside the translocator pore. Additionally, several ATs can be released through the assistance of other proteases or ATs, such as omptins and NalP, respectively.

3.2 AT Classification

In 1987, the peculiarity of the secretion mechanism of the IgA1 protease from *Neisseria meningitidis* was described (Pohlner et al. 1987); defining the first AT protein and soon after, establishing the T5SS (Henderson et al. 2004). Since then, a large number of other AT molecules have been identified and many of them with a distinct domain organization and peculiar structural characteristics, which have prompted researchers to further classify them into five subcategories designated Va-Ve (Fig. 10.2).

3.3 Va: Classical Monomeric ATs

The Va subcategory comprises the widespread classic monomeric autotransporters, whose cognates display a basic AT structure comprising of a classical N-terminal passenger and a C-terminal translocator domain (Pohlner et al. 1987). Two distinctive groups can be recognized in the classical monomeric ATs; those whose passengers are secreted into the extracellular milieu, such as the IgA1 proteases, the serine protease autotransporters of *Enterobacteriaceae* (SPATE) and the subtilisin-like serine proteases; and those whose passenger domains are retained and exposed on the bacterial surface such as the adhesins collectively called self-associating ATs (SAAT) (Klemm et al. 2006).

3.3.1 The IgA1 Proteases

The IgA proteases of *Neisseria* and *Haemophilus* were the first monomeric ATs identified (Pohlner et al. 1987). However, to date no IgA protease has been recognized in pathogenic *E. coli* and they seem mainly confined to respiratory pathogens. Although numerous functions have been attributed to the IgA1 proteases, their roles in pathogenesis remain obscure. In vitro studies have shown to cleave the hinge region of human secretory IgA1 and the major integral membrane glycoprotein of lysosomes, LAMP1; hence, suggesting an IgA protease role in bacterial colonization on mucosal surfaces and in intracellular replication (Pohlner et al. 1987; Henderson et al. 2004).

3.3.2 SPATES

Most secreted monomeric ATs are members of the SPATE family, which belong to the trypsin-like superfamily of serine proteases (Ruiz-Perez and Nataro 2014). The term SPATE was conceived to enclose serine proteases produced by the *Enterobacteriaceae* with similar characteristics including: (1) presence of an extended signal peptide (48–59 aa); (2) the presence of a catalytic triad formed by H, D, and S residues embedded in the motif GDSGS; and (3) the existence of a cleavage site (two consecutive asparagines; N-N), which detaches the passenger domain from the translocator domain (Ruiz-Perez and Nataro 2014).

SPATEs are secreted by species of *Shigella*, *Salmonella*, *Edwardsiella*, *Citrobacter,* and all recognized *E. coli* pathotypes associated with enteric/diarrheal disease, and extra-intestinal *E. coli* pathogens (ExPEC), such as uropathogenic *E. coli* (UPEC) and septicemic *E. coli*, which are responsible for urinary tract infections (UTIs) and sepsis/meningitis, respectively (Ruiz-Perez and Nataro 2014). SPATEs have been divided into class-1 and class-2 based on structural differences and biological effects (Ruiz-Perez and Nataro 2014). Class-1 SPATEs studied so far display similar substrate specificity, consistent cytotoxic effects on cultured cells, and enterotoxin activity on intestinal tissues (Navarro-Garcia et al. 2010; Ruiz-Perez and Nataro 2014), whereas most class-2 SPATEs exhibit a lectin-like activity with predilection to degrade a variety of mucins, including leukocyte surface O-glycoproteins and soluble host proteins, resulting in an advantage for mucosal colonization and immune modulation (Ruiz-Perez et al. 2011; Abreu et al. 2016). Archetype SPATEs are exemplified by Pet (Plasmid encoded toxin), predominantly distributed in EAEC isolates (Navarro-Garcia et al. 2010), and Pic (Protease involved in colonization) widely spread in EAEC, UPEC, *Shigella flexneri,* and *Citrobacter* strains (Ruiz-Perez and Nataro 2014).

3.3.3 Subtilase-Like ATs

The other family of secreted monomeric autotransporters is grouped into the subtilase-like autotransporters, which share a catalytic site common for type S8 peptidases (Henderson et al. 2004). Members of this family bear the conserved domain pfam00082 and are best characterized in *Bacillus subtilus* and other pathogenic Gram-negative species, but not been found in *E. coli*.

3.3.4 Self-Associating Autotransporters

TibA from ETEC, AIDA from DAEC, and Ag43 present in several *E. coli* pathotypes are the most extensively studied surface exposed monomeric autotransporters, collectively known as self-associating autotransporters (SAAT) by virtue of their cell-cell interacting properties (Klemm et al. 2006). AIDA is the archetype

SAAT and the first surface-exposed monomeric AT identified, which mediates adherence to human cells. Contiguous to *aida-I* is the *aah* gene, which encodes a heptosyltransferase that modifies the AIDA adhesin by addition of heptose residues (Benz and Schmidt 2001). AIDA, TibA, Ag43, and many other members of this family undergo extensive post-translational glycosylation of the passenger domain, which is crucial for their adherence to host cells and the ability to colonize the mouse intestine (Lu et al. 2014). The recently solved structure of the heptosyltransferase TibC (Aah homologue) reveals an extraordinary hyperglycosylation machinery in which six autotransporter substrates are glycosylated simultaneously (Yao et al. 2014).

3.4 Vb: Two-Partner System (TPS)

The Vb subcategory embraces the two-partner secretion system (TPS), in which the passenger and the translocator domain harboring polypeptide-transport-associated (POTRA) motifs are encoded by distinct genes (Jacob-Dubuisson et al. 2013) (Fig. 10.2). TPS are produced as two separate proteins, each containing an N-terminal signal sequence, which allows inner-membrane translocation to the periplasm (Jacob-Dubuisson et al. 2013). The passenger and translocation proteins are known as TpsA and TspB, respectively. TpsA are large proteins, share significant sequence similarity in a 250 aa N-terminal region called TPS domain, and adopt a solenoid or β-helical conformation (Jacob-Dubuisson et al. 2013). TpsB partners are about 60 kDa proteins, composed of a periplasmic moiety formed by two successive POTRA domains (Sanchez-Pulido et al. 2003), which are involved in the recognition of the TpsA passenger proteins and a C-terminal moiety that is embedded in the OM to conform the β-barrel pore for TpsA translocation. The TpsA polypeptide progressively crosses the OM and folds at the cell surface where it can persist non-covalently attached or released into the extracellular milieu after translocation (Jacob-Dubuisson et al. 2013).

TpsA proteins exhibit a large functional diversity, which includes adherence, proteolysis, inhibition of phagocytosis, systemic dissemination of bacteria, intracellular survival, bacterial aggregation, biofilm formation, hemolysis/cytolysis, contact-dependent growth inhibition, and iron acquisition, which contribute to bacterial fitness or virulence (Anderson et al. 2012).

The TPS identified in pathogenic *E. coli* so far include those related to contact-dependent growth inhibition (CDI) and adherence. The CDI was identified in *E. coli* EC93, a dominant isolate from rat intestine. ETEC expresses the 170-kDa glycosylated adhesin EtpA encoded in the *etpBAC* TPS operon, whose main role is to contribute to intestinal colonization (Sahl et al. 2011).

The EHEC O157:H7 genome exhibits a putative TPS system designated OtpA and OtpB of unknown function, but found almost exclusively in the Shiga toxin-producing serotypes associated with hemolytic uremic syndrome (Shen et al. 2005).

3.5 Vc: Trimeric Autotransporter Adhesins

Trimeric autotransporters adhesins (TAA), also known as the Oca family, are characterized by the presence of three passenger domains fused to the one-third segment of a fully functional C-terminal translocator domain, allowing secretion of trimeric polypeptides (Henderson et al. 2004) (Fig. 10.2). TAAs are produced as a single polypeptide, but they diverge from classical ATs in the size of the β-barrel translocation domain. Classical AT β-barrels comprise 12 β-strands, while TAAs have only four β-strands (70–100 aa) (Roggenkamp et al. 2003). Consequently, to become functional, TAA translocation domains assemble in 12-stranded β-barrel homotrimer at the OM, which convey stability of the β-barrel and functionality of the passenger domain. Solved structure of prototypical TAAs has revealed three distinct regions within the passenger domain: An N-terminal head, a connector/neck stalk region that is variable in length, and a C-terminal anchor domain (Roggenkamp et al. 2003). The N-terminal head structure differs between TAAs and is primarily involved in the adhesion properties of the protein (Roggenkamp et al. 2003). The stalk is a fibrous, highly repetitive structure that works as spacers to project the head domain away from the bacterial surface and can convey protection against host defenses such as serum resistance and antibody binding. The neck functions as a short adapter between the large globular head and the narrow stalk domain, partly explaining the stability of trimeric proteins (Roggenkamp et al. 2003).

TAA are virulence factors with the ability to mediate adherence to eukaryotic cells or extracellular matrix (ECM) proteins such as collagen and laminin. Only few TAAs have been described in *E. coli* including Saa, Eib, UpaG, and EhaG. The immunoglobulin-(Ig)-binding proteins from *E. coli* (Eibs) are relatively common in LEE-negative, STEC strains (Merkel et al. 2010), comprising six characterized members, called EibA-C-D-E-F-G (Merkel et al. 2010). Eibs are receptors for IgAs and IgGs and bind to the Fc portion of Igs (Leo and Goldman 2009), confer serum resistance, and are involved in adherence to epithelial cells.

The *saa* gene encodes an auto-agglutinating adhesion-designated Saa, found in the megaplasmid of a LEE-negative STEC O113:H21 (98NK2), responsible for an HUS outbreak (Paton et al. 2001). Homologues of *saa* were found in several unrelated LEE-negative STEC serotypes, which were also isolated from patients with HUS. However, no significant association was found between the *saa* gene and STEC isolated from patients with HUS (Jenkins et al. 2003). The *saa*-positive strains were detected more frequently in STEC strains from bovines than in those from humans, suggesting that Saa may have a role in attachment to the bovine gut (Jenkins et al. 2003).

UpaG is a TAA identified in ExPEC, which promotes cell aggregation and biofilm formation on abiotic surfaces and allows specific adhesion of UPEC CFT073 to human bladder epithelial cells by binding to extracellular matrix (ECM) proteins, such as fibronectin and laminin (Valle et al. 2008).

EhaG found in *E. coli* O157:H7 is a positional orthologue of UpaG, but they diverge significantly within the passenger domain. EhaG is localized at the bacterial cell surface and promotes cell aggregation, biofilm formation, and adherence

to a range of extra-cellular matrix (ECM) proteins. Similar to EhaG, which mediates specific adhesion to colorectal epithelial cells, UpaG promotes specific binding to bladder epithelial cells (Totsika et al. 2012).

3.6 Vd: Fusion of Classical and TPS ATs

Autotransporters in this subcategory resemble a fusion between the Va monomeric ATs and the Vb two partner system autotransporters (Fig. 10.2). The archetype member of this subcategory is the patatin-like protein from *Pseudomonas aeruginosa*, named PlpD (Salacha et al. 2010), which has more than 200 PlpD orthologues among pathogenic and environmental bacteria. However, this AT subcategory has not been found in *E. coli*. The classifying feature of this subcategory is that the passenger domain is connected to the translocator β-barrel with a POTRA domain (Sanchez-Pulido et al. 2003; Salacha et al. 2010). The passenger domain of PlpD is a lipolytic enzyme released by an autocatalytic reaction after translocation. Given that the other patatin ExoU from *P. aeruginosa* is a major cytotoxin, it is likely that PlpD is also a virulence factor. Nevertheless, PlpD homologues have been also found in environmental bacterial species, suggesting its involvement in other bacterial functions (Salacha et al. 2010).

3.7 Ve: Inverse ATs

The archetype ATs of this subcategory are the invasin of enteropathogenic *Yersinia* strains and intimin of EHEC and EPEC strains (Schmidt 2010). Both proteins belong to a large family of adhesins widely distributed in Gram-negative bacteria and are central virulence factors mediating binding to host cells. Although these proteins were identified a long time ago, only recently, they were recognized as a new variety of autotransporter proteins, in which contrary to classical ATs, Ve has an inverted topology with the passenger domain located in the C-terminal and the translocation unit in the N-terminal of the molecule; hence the term inverse autotransporter (Fig. 10.2).

3.7.1 Intimin

This protein is a 94 kDa OM adhesin encoded by *eaeA* gene in the LEE, found in various diarrheagenic pathogens which cause A/E lesions including EPEC, EHEC, *C. rodentium,* and *Hafnia alvei* (Schmidt 2010). Intimin is expressed on the bacterial cell surface where it can bind to its receptor Tir (translocated intimin receptor). As the name suggests, intimin mediates “intimately” adherence to the epithelium and cause the formation of actin pedestals and the disruption of the microvilli on the enterocyte surface.

Several studies have shown the pivotal role of intimin in EPEC and EHEC virulence. For example, mutations in the *eaeA* gene result in loss of ability to cause A/E lesions and attenuation of the pathogen in infected volunteers and animal models (Schmidt 2010).

3.7.2 Invasins

Invasin is encoded by the *invA* gene in *Yersinia* species, but *invA* homologues are also found in *Salmonella* and *Edwardsiella* species. Invasin binds to a subset of β1-integrin receptors located on the apical membrane of intestinal M-cells, thereby facilitating invasion of cells and the lymphatic system underneath. Analysis of sequenced genomes has revealed that intimin and invasin are widespread in *Proteobacteria*, *Planctomycetes*, *Cyanobacteria,* and *Chlamydiae* (Fairman et al. 2012).

In summary, significant advances have been made to understand the T5SS biogenesis, their biological functions, and their roles in pathogenesis. The identification of new ATs, their biological functions, in vivo targets, cell receptors, and effects in animal models have provided insights into the virulence roles of ATs in the context of disease. Moreover, because of the simplicity of this secretion mechanism with respect to the other bacterial secretion systems, ATs are the vector of choice for cell surface display of heterologous proteins for biotechnological applications, such as the screening of protein libraries, whole cell biocatalysis, and live vaccine development.

4 The Type 6 Secretion System (T6SS)

T6SS is the most recently described specialized secretion system in Gram-negative bacteria. It is found widely distributed in proteobacteria, where their gene clusters may be found in several copies on the chromosome. Initially, it was thought as secretion system dedicated to virulence toward eukaryotic host cells as recent data have shown a pivotal role in pathogenesis. However, only a limited number of T6SSs have been shown to be directly responsible for pathogenesis. Nevertheless, it seems that the most important role of T6SS is in bacterial competition by killing neighboring non-immune bacterial cells through secretion of anti-bacterial proteins directly into the periplasm of the target cells upon cell-to-cell contact.

4.1 Secretion Apparatus (Organelle)

T6SS is a double-membrane-spanning secretion apparatus, which delivers substrates directly from the bacterial cytoplasm to the extracellular space or into a target cell (Fig. 10.3). The substrates are transported by a chaperone-usher pathway

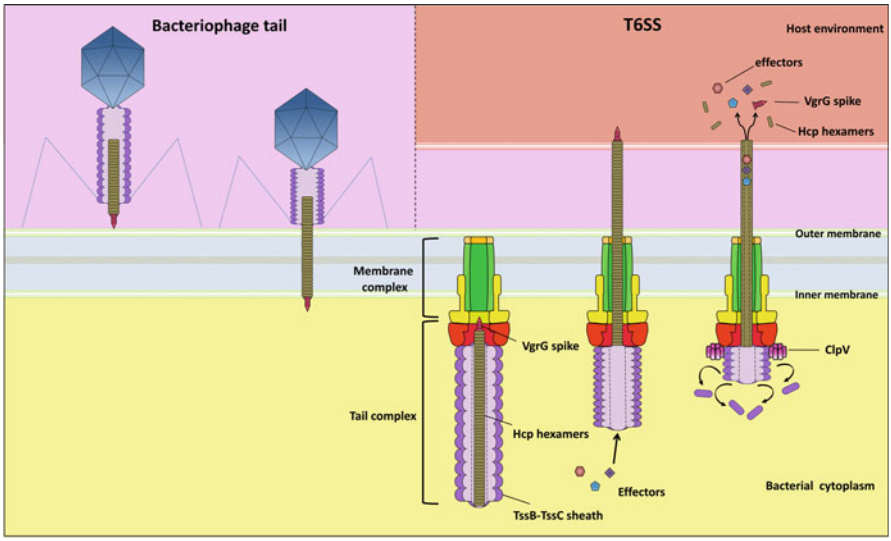

Fig. 10.3 The T6SS is composed of a membrane complex and a tail complex. TssJ-TssL-TssM makes the membrane complex and is connected to the TssB-TssC tail sheath and the hemolysin co-regulated protein (Hcp) inner tube through the baseplate (composed of TssK, TssE, and VgrG). Effectors are recruited to the spike–tube complex through the extension domains of VgrG and/or PAAR-repeat proteins and through incorporation into the hemolysin co-regulated protein (Hcp) tube. An unknown extracellular signal triggers sheath contraction, which leads to the ejection of the spike–tube complex across the target membrane, thereby delivering effector proteins into the cell. The ATPase ClpV disassembles the contracted TssB-TssC sheath, which enables a new T6SS complex to be reassembled from the released subunits

and can be secreted partially or entirely folded. Thirteen subunits, called core components, are believed to form the minimal apparatus, which in several cases are supplemented with additional proteins (Cascales and Cambillau 2012). T6SS components are generally encoded in clustered genes averaging 20 kb, and in many instances, multiple T6SS gene clusters can be identified in a single organism (Gode-Potratz and McCarter 2011).

The T6SS machinery consists of two main membrane complex, which comprises inner membrane (IM) proteins that are homologous to components of the T4SS, and a tail complex that contains elements that are evolutionarily related to contractile bacteriophage tails (Leiman et al. 2009). The minimal T6SS membrane complex is composed of TssJ, TssL, and TssM. TssM is an IM protein that connects TssJ, an outer membrane (OM) lipoprotein, to the IM TssL protein. TssK is a cytoplasmic subunit that connects the TssJ-TssL-TssM membrane complex to the T6SS tail, and it has, therefore, been proposed to be a component of the T6SS baseplate (Zoued et al. 2013). The tail sheath (TssB-TssC complex) is a long tubular structure that is roughly perpendicular to the membrane and extends deep into the bacterial cell cytoplasm. Inside the tail sheath is an inner tube comprised of polymerized hemolysin co-regulated protein (Hcp) (Leiman et al. 2009). This component is

essential to the T6SS function and forms a tube of stacked hexamers in vitro. Six Hcp molecules assemble to form 80–90 Å wide hexameric rings stabilized by an extension acting as an inter-subunit belt. The Hcp hexamers have been shown to assemble as tubes with a diameter of 35–40 Å, which may therefore accommodate a small folded protein or unfolded/partly folded protein. The VgrG (valine-glycine repeat G) trimer forms a spike in the center of the baseplate complex and is thought to function as a nucleation platform for proper assembly of the T6SS tail tube (Leiman et al. 2009).

Similar to the bacteriophage T4 sheath, contraction of the TssB/TssC tubule propels the Hcp tube toward the external milieu. This contraction might be provoked by the ClpV AAA+ ATPase that has been shown to depolymerize TssBC tubules. The ClpV chaperone, or TssH in the T6SS nomenclature, has been shown to play a crucial role in the T6SS assembly mechanism. When the soluble proteins TssB and TssC are mixed, they assemble spontaneously in long tubes that may prevent the translocation of the monomers into the periplasm to form the tube sheath (Fig. 10.3). Then, the ClpV ATPase might therefore act at two steps during T6SS assembly and function by: depolymerizing the TssBC tubules in the cytosol allowing their transport into the periplasm, where TssB and TssC will polymerize to form the sheath-like structure, and by depolymerizing the putative sheath to provide the energy required for its contraction (Aubert et al. 2010).

TssE is thought to initiate the polymerization of the sheath by an arm-exchange mechanism with the first row. The other components of the base plate are likely being the essential core components, TssAFGK. TssK is an intrinsically cytoplasmic inner membrane-associated trimeric protein, which can form higher order oligomers (English et al. 2014). Interactions of TssK with TssL, TssC, and Hcp in *E. coli* suggest that it may have a role in connecting the T6SS bacteriophage tail-like and membrane complexes (English et al. 2014). Using time lapse fluorescence microscopy, it was shown that in EAEC, TssA binds first to the type VI secretion membrane core complex and then initiates tail polymerization, while remaining at the tip of the growing structure, where it incorporates more tube and sheath blocks (Zoued et al. 2016). Therefore, TssA protein was proposed to be the component responsible of priming and coordinating tail tube and sheath biogenesis (Zoued et al. 2016).

By analogy with the phage tail structure and its assembly pathway, the Hcp tube is thought to assemble onto the VgrG trimer and to propel it towards the target cell as a result of TssBC contraction. The needle-shaped β-helical domain of VgrG will help to penetrate the target cell. A number of VgrG proteins carry C-terminal extensions located downstream the β-prism, which may function as effector domains; these VgrGs have been named “specialized VgrGs.” An additional protein, called PAAR, usually encoded within T6SS forms a conical structure that caps the extremity of VgrG, sharpening the tip of the spike (Shneider et al. 2013). PAAR is, therefore, secreted in a VgrG-dependent manner. It was proposed that PAAR may function as a connector between VgrG and effector proteins, given that a number of PAAR proteins are fused to putative effector domains (Shneider et al. 2013).

4.2 T6SS in E. coli

The leading research regarding T6SS in *E. coli* has been made in EAEC and in avian pathogenic *E. coli* (APEC). EAEC possess two T6SS clusters designated *sci-1* and *sci-2* (Dudley et al. 2006). Whereas, three distinct T6SS loci exist in APEC, in which more than 85 % of T6SSs loci-containing APEC strains belong to the virulent phylogenetic groups D and B2, indicating that T6SSs might contribute to APEC pathogenicity. In fact, recent studies show that APEC T6SS1 is involved in colonization and proliferation in systemic infections and T6SS2 is responsible for intramacrophage survival, cytokine and chemokine release, and host cell apoptosis (Zhou et al. 2012).

Although not yet functionally characterized, there are more than ten T6SS orthologues in other *E. coli* species, including EHEC strains EDL933 and Sakai, EPEC strain B171, UPEC strains 536, UTI89, and CFT073, and neonatal meningitis *E. coli* (NMEC) strains S88 and IHE3034 (Aschtgen et al. 2010).

4.3 Regulation of T6SS

Common trends in the regulatory mechanisms of T6SS include quorum sensing (QS), changes in temperature and pH, and two-component regulatory systems (TCSs) (Gode-Potratz and McCarter 2011). Environmental elements such as the concentration of iron, phosphate, and magnesium have been also implicated in T6SS regulation (Lv et al. 2012). While these mechanisms exert tight control over the T6SS, other species possess constitutively active T6SSs. The Sci-1 T6SS of EAEC is regulated at the transcriptional level by iron availability through a pathway involving DNA adenine methyltransferase (Dam)-catalyzed methylation and Fur repression (Brunet et al. 2011). Fur protein is an essential modulator of iron-dependent gene expression in bacteria. Therefore, Sci-1 activation mediated by Fur depression could be the consequence of environmental signals such as oxidative stress or iron starvation. On the other hand, expression of the Sci-2 gene cluster, encoded with Sci-1 within the *pheU* pathogenicity island, has been shown to be controlled by the AraC transcriptional activator AggR, which is a master regulator of EAEC virulence.

4.4 Protein Translocation into Prokaryotic and Eukaryotic Cells

The broad range of toxins delivered by T6SS reflects the diversity of the cell targets and biological activities associated with this system. Recent studies have highlighted two main types of effectors: those fused to structural components as “specialized”

VgrG effectors, or those non-covalently attached (cargo effectors) to structural components (Durand et al. 2014). In both cases, effectors are associated with components of the expelled Hcp-VgrG-PAAR structure, suggesting that multiple effectors decorating this puncturing device could be simultaneously delivered into a target cell in one lethal puncture (Shneider et al. 2013).

4.4.1 Effectors That Target Eukaryotic Cells

Two types of T6SS effector that target eukaryotic cells have been identified: C-terminal domains (CTD) toxins borne by specialized VgrG proteins and independent toxins. VgrG protein is propelled towards the target cell during T6SS function, directly delivering the CTD into the host cell cytosol after membrane perforation (Chang and Kim 2015). In the cytosol, CTD catalyze covalent cross-linking of G-actin in an ATP-dependent manner, causing aggregation of actin molecules and preventing host cell cytoskeleton rearrangements, which disables phagocytosis of macrophages (Aschtgen et al. 2010).

4.4.2 Effectors Targeting Bacterial Cells

These effectors can be classified in three categories: cell wall-degrading enzymes (murein hydrolases), membrane-targeting proteins (phospholipases and pore-forming), and nucleases. It was recently shown that lethal attacks by T6SS result in induction of *soxS* and enhanced ROS levels in *E. coli*, similar to the effect triggered by P1vir phage and polymyxin B. A common feature of these effectors is that they are produced concomitantly with specific immunity proteins that bind to the cognate toxin, preventing autointoxication (Miyata et al. 2013).

It is now known that the T6SS is used to deliver antibacterial toxins directly inside neighboring bacterial cells. This killing mechanism has been recorded in real time by using time-lapse fluorescence microscopy (Brunet et al. 2013). In co-cultures, prey cells are killed upon contact with predator cells (bearing T6SS), in which sheath contraction correlates with nearby cell fading. More striking is that prey lysis occurs within minutes after sheath contraction (Brunet et al. 2013). Interestingly, bacteria bearing T6SS are able to counterattack the assault of other aggressive T6SS-bearing bacteria. In the case of *P. aeruginosa*, T6SS-dependent killing of other bacterial species is greatly stimulated by the T6SS in the prey species, suggesting that assembly of the T6SS organelle and lethal counterattack are regulated by a signal originated from the point of attack of the T6SS of a second aggressive T6SS (+) bacterial cell.

Beside the role of T6SS in interbacterial competition, it has been shown that different isolates produce diverse sets of toxin effectors and, therefore, competition occurs among multiple strains of the same species (intraspecific competition). This ability is responsible for the limited growth observed between non-isogenic strains in several species.

4.5 Remarkable Conclusions

Since its conception, the T6SS became a highly exciting area of research. Several significant discoveries in archetype bacteria have delighted and encouraged researchers to investigate their roles in other pathogens, which has expanded the knowledge rapidly. All T6SS topics have been fascinating, from dissecting the architecture similarities among T6SS and contractile bacteriophage tails, to the mechanism of action of effector proteins on mammalian cells and competing bacteria, not to mention their mechanism of regulation and silencing, how they are triggered to counterattack the insults of other aggressive bacteria and how immunity proteins evolved to protect against T6SS effectors. There is no doubt that a remarkable progress has been made regarding the overall architecture and biogenesis; however, new investigations are required to understand the roles of T6SS and its effectors in models in vivo, in the context of disease; topics that we will certainly see in the next years.

References

Abreu AG, Abe CM, Nune KO, Moraes CT, Chavez-Duenas L, Navarro-Garcia F, Barbosa AS, Piazza RM, Elias WP (2016) The serine protease Pic as a virulence factor of atypical enteropathogenic *Escherichia coli*. Gut Microbes 7(2):115–125

Alto NM, Shao F, Lazar CS, Brost RL, Chua G, Mattoo S, McMahon SA, Ghosh P, Hughes TR, Boone C, Dixon JE (2006) Identification of a bacterial type III effector family with G protein mimicry functions. Cell 124(1):133–145

Anderson MS, Garcia EC, Cotter PA (2012) The *Burkholderia* bcpAIOB genes define unique classes of two-partner secretion and contact dependent growth inhibition systems. PLoS Genet 8(8), e1002877

Arbeloa A, Garnett J, Lillington J, Bulgin RR, Berger CN, Lea SM, Matthews S, Frankel G (2010) EspM2 is a RhoA guanine nucleotide exchange factor. Cell Microbiol 12(5):654–664

Aschtgen M-S, Gavioli M, Dessen A, Lloubès R, Cascales E (2010) The SciZ protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the cell wall. Mol Microbiol 75:886–899

Ashida H, Ogawa M, Mimuro H, Kobayashi T, Sanada T, Sasakawa C (2011) *Shigella* are versatile mucosal pathogens that circumvent the host innate immune system. Curr Opin Immunol 23(4):448–455

Aubert D, MacDonald DK, Valvano MA (2010) BcsKC is an essential protein for the type VI secretion system activity in *Burkholderia cenocepacia* that forms an outer membrane complex with BcsLB. J Biol Chem 285:35988–35998

Batchelor M, Guignot J, Patel A, Cummings N, Cleary J, Knutton S, Holden DW, Connerton I, Frankel G (2004) Involvement of the intermediate filament protein cytokeratin-18 in actin pedestal formation during EPEC infection. EMBO Rep 5(1):104–110

Benz I, Schmidt MA (2001) Glycosylation with heptose residues mediated by the aah gene product is essential for adherence of the AIDA-I adhesin. Mol Microbiol 40(6):1403–1413

Bingle LE, Constantinidou C, Shaw RK, Islam MS, Patel M, Snyder LA, Lee DJ, Penn CW, Busby SJ, Pallen MJ (2014) Microarray analysis of the Ler regulon in enteropathogenic and enterohaemorrhagic *Escherichia coli* strains. PLoS One 9(1), e80160

Blasche S, Mortl M, Steuber H, Siszler G, Nisa S, Schwarz F, Lavrik I, Gronewold TM, Maskos K, Donnenberg MS, Ullmann D, Uetz P, Kogl M (2013) The *E. coli* effector protein NleF is a caspase inhibitor. PLoS One 8(3), e58937

Blocker A, Jouihri N, Larquet E, Gounon P, Ebel F, Parsot C, Sansonetti P, Allaoui A (2001) Structure and composition of the *Shigella flexneri* "needle complex", a part of its type III secreton. Mol Microbiol 39(3):652–663

Brunet YR, Bernard CS, Gavioli M, Lloubès R, Cascales E (2011) An epigenetic switch involving overlapping *fur* and DNA methylation optimizes expression of a type VI secretion gene cluster. PLoS Genet 7, e1002205

Brunet YR, Espinosa L, Harchouni S, Mignot T, Cascales E (2013) Imaging type VI secretion-mediated bacterial killing. Cell Rep 3(1):36–41

Bulgin RR, Arbeloa A, Chung JC, Frankel G (2009) EspT triggers formation of lamellipodia and membrane ruffles through activation of Rac-1 and Cdc42. Cell Microbiol 11(2):217–229

Bustamante VH, Villalba MI, Garcia-Angulo VA, Vazquez A, Martinez LC, Jimenez R, Puente JL (2011) PerC and GrlA independently regulate Ler expression in enteropathogenic *Escherichia coli*. Mol Microbiol 82(2):398–415

Campellone KG, Rankin S, Pawson T, Kirschner MW, Tipper DJ, Leong JM (2004) Clustering of Nck by a 12-residue Tir phosphopeptide is sufficient to trigger localized actin assembly. J Cell Biol 164(3):407–416

Cascales E, Cambillau C (2012) Structural biology of type VI secretion systems. Philos Trans R Soc Lond B Biol Sci 367:1102–1111

Chang JH, Kim Y-G (2015) Crystal structure of the bacterial type VI secretion system component TssL from *Vibrio cholerae*. J Microbiol 53:32–37

Cheng HC, Skehan BM, Campellone KG, Leong JM, Rosen MK (2008) Structural mechanism of WASP activation by the enterohaemorrhagic *E. coli* effector EspF(U). Nature 454(7207):1009–1013

Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol 13(6):343–359

Creasey EA, Friedberg D, Shaw RK, Umanski T, Knutton S, Rosenshine I, Frankel G (2003) CesAB is an enteropathogenic *Escherichia coli* chaperone for the type-III translocator proteins EspA and EspB. Microbiology 149(Pt 12):3639–3647

Crepin VF, Shaw R, Abe CM, Knutton S, Frankel G (2005) Polarity of enteropathogenic *Escherichia coli* EspA filament assembly and protein secretion. J Bacteriol 187(8):2881–2889

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB (2013) Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26(4):822–880

Deng W, Puente JL, Gruenheid S, Li Y, Vallance BA, Vazquez A, Barba J, Ibarra JA, O'Donnell P, Metalnikov P, Ashman K, Lee S, Goode D, Pawson T, Finlay BB (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. Proc Natl Acad Sci U S A 101(10):3597–3602

Deng W, Yu HB, de Hoog CL, Stoynov N, Li Y, Foster LJ, Finlay BB (2012) Quantitative proteomic analysis of type III secretome of enteropathogenic *Escherichia coli* reveals an expanded effector repertoire for attaching/effacing bacterial pathogens. Mol Cell Proteomics 11(9):692–709

Doyle MT, Tran EN, Morona R (2015) The passenger-associated transport repeat promotes virulence factor secretion efficiency and delineates a distinct autotransporter subtype. Mol Microbiol 97(2):315–329

Drobnak I, Braselmann E, Clark PL (2015) Multiple driving forces required for efficient secretion of autotransporter virulence proteins. J Biol Chem 290(16):10104–10116

Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP (2006) Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative *Escherichia coli*. Mol Microbiol 61(5):1267–1282

Durand E, Cambillau C, Cascales E, Journet L (2014) VgrG, Tae, Tle, and beyond: the versatile arsenal of Type VI secretion effectors. Trends Microbiol 22:498–507

English G, Byron O, Cianfanelli FR, Prescott AR, Coulthurst SJ (2014) Biochemical analysis of TssK, a core component of the bacterial Type VI secretion system, reveals distinct oligomeric states of TssK and identifies a TssK-TssFG subcomplex. Biochem J 461:291–304

Fairman JW, Dautin N, Wojtowicz D, Liu W, Noinaj N, Barnard TJ, Udho E, Przytycka TM, Cherezov V, Buchanan SK (2012) Crystal structures of the outer membrane domain of intimin and invasin from enterohemorrhagic *E. coli* and enteropathogenic *Y. pseudotuberculosis*. Structure 20(7):1233–1243

Garmendia J, Phillips AD, Carlier MF, Chong Y, Schuller S, Marches O, Dahan S, Oswald E, Shaw RK, Knutton S, Frankel G (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. Cell Microbiol 6(12):1167–1183

Gode-Potratz CJ, McCarter LL (2011) Quorum sensing and silencing in *Vibrio parahaemolyticus*. J Bacteriol 193:4224–4237

Gruber CC, Sperandio V (2015) Global analysis of posttranscriptional regulation by GlmY and GlmZ in enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 83(4):1286–1295

Hayes CS, Aoki SK, Low DA (2010) Bacterial contact-dependent delivery systems. Annu Rev Genet 44:71–90

Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D (2004) Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68(4):692–744

Huang Z, Sutton SE, Wallenfang AJ, Orchard RC, Wu X, Feng Y, Chai J, Alto NM (2009) Structural insights into host GTPase isoform selection by a family of bacterial GEF mimics. Nat Struct Mol Biol 16(8):853–860

Hueck CJ (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 62(2):379–433

Ide T, Laarmann S, Greune L, Schillers H, Oberleithner H, Schmidt MA (2001) Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. Cell Microbiol 3(10):669–679

Iyoda S, Watanabe H (2005) ClpXP protease controls expression of the type III protein secretion system through regulation of RpoS and GrlR levels in enterohemorrhagic *Escherichia coli*. J Bacteriol 187(12):4086–4094

Iyoda S, Koizumi N, Satou H, Lu Y, Saitoh T, Ohnishi M, Watanabe H (2006) The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic *Escherichia coli*. J Bacteriol 188(16):5682–5692

Jacob-Dubuisson F, Guerin J, Baelen S, Clantin B (2013) Two-partner secretion: as simple as it sounds? Res Microbiol 164(6):583–595

Jenkins C, Perry NT, Cheasty T, Shaw DJ, Frankel G, Dougan G, Gunn GJ, Smith HR, Paton AW, Paton JC (2003) Distribution of the saa gene in strains of Shiga toxin-producing *Escherichia coli* of human and bovine origins. J Clin Microbiol 41(4):1775–1778

Jimenez R, Cruz-Migoni SB, Huerta-Saquero A, Bustamante VH, Puente JL (2010) Molecular characterization of GrlA, a specific positive regulator of ler expression in enteropathogenic *Escherichia coli*. J Bacteriol 192(18):4627–4642

Kawamoto A, Morimoto YV, Miyata T, Minamino T, Hughes KT, Kato T, Namba K (2013) Common and distinct structural features of *Salmonella* injectisome and flagellar basal body. Sci Rep 3:3369

Kendall MM, Gruber CC, Parker CT, Sperandio V (2012) Ethanolamine controls expression of genes encoding components involved in interkingdom signaling and virulence in enterohemorrhagic *Escherichia coli* O157:H7. mBio 3(3):e00050. doi:10.1128/mBio.00050-12

Klemm P, Vejborg RM, Sherlock O (2006) Self-associating autotransporters, SAATs: functional and structural similarities. Int J Med Microbiol 296(4–5):187–195

Kubori T, Matsushima Y, Nakamura D, Uralil J, Lara-Tejero M, Sukhan A, Galan JE, Aizawa SI (1998) Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science 280(5363):602–605

Leiman PG, Basler M, Ramagopal UA, Bonanno JB, Sauder JM, Pukatzki S, Burley SK, Almo SC, Mekalanos JJ (2009) Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. Proc Natl Acad Sci U S A 106:4154–4159

Leo JC, Goldman A (2009) The immunoglobulin-binding Eib proteins from *Escherichia coli* are receptors for IgG Fc. Mol Immunol 46(8–9):1860–1866

Leyton DL, Johnson MD, Thapa R, Huysmans GH, Dunstan RA, Celik N, Shen HH, Loo D, Belousoff MJ, Purcell AW, Henderson IR, Beddoe T, Rossjohn J, Martin LL, Strugnell RA,

Lithgow T (2014) A mortise-tenon joint in the transmembrane domain modulates autotransporter assembly into bacterial outer membranes. Nat Commun 5:4239
Lommel S, Benesch S, Rottner K, Franz T, Wehland J, Kuhn R (2001) Actin pedestal formation by enteropathogenic *Escherichia coli* and intracellular motility of Shigella flexneri are abolished in N-WASPdefective cells. EMBO reports 2(9):850–857. doi:10.1093/embo-reports/kve197
Lu Q, Yao Q, Xu Y, Li L, Li S, Liu Y, Gao W, Niu M, Sharon M, Ben-Nissan G, Zamyatina A, Liu X, Chen S, Shao F (2014) An iron-containing dodecameric heptosyltransferase family modifies bacterial autotransporters in pathogenesis. Cell Host Microbe 16(3):351–363
Luo W, Donnenberg MS (2011) Interactions and predicted host membrane topology of the enteropathogenic *Escherichia coli* translocator protein EspB. J Bacteriol 193(12):2972–2980
Lv Y, Xiao J, Liu Q, Wu H, Zhang Y, Wang Q (2012) Systematic mutation analysis of two-component signal transduction systems reveals EsrA-EsrB and PhoP-PhoQ as the major virulence regulators in *Edwardsiella tarda*. Vet Microbiol 157:190–199
Ma C, Wickham ME, Guttman JA, Deng W, Walker J, Madsen KL, Jacobson K, Vogl WA, Finlay BB, Vallance BA (2006) *Citrobacter rodentium* infection causes both mitochondrial dysfunction and intestinal epithelial barrier disruption *in vivo*: role of mitochondrial associated protein (Map). Cell Microbiol 8(10):1669–1686
Maresca M, Miller D, Quitard S, Dean P, Kenny B (2005) Enteropathogenic *Escherichia coli* (EPEC) effector-mediated suppression of antimicrobial nitric oxide production in a small intestinal epithelial model system. Cell Microbiol 7(12):1749–1762
Matsuzawa T, Kuwae A, Yoshida S, Sasakawa C, Abe A (2004) Enteropathogenic *Escherichia coli* activates the RhoA signaling pathway via the stimulation of GEF-H1. EMBO J 23(17): 3570–3582
Merkel V, Ohder B, Bielaszewska M, Zhang W, Fruth A, Menge C, Borrmann E, Middendorf B, Muthing J, Karch H, Mellmann A (2010) Distribution and phylogeny of immunoglobulin-binding protein G in Shiga toxin-producing *Escherichia coli* and its association with adherence phenotypes. Infect Immun 78(8):3625–3636
Mitra A, Fay PA, Morgan JK, Vendura KW, Versaggi SL, Riordan JT (2012) Sigma factor N, liaison to an ntrC and rpoS dependent regulatory pathway controlling acid resistance and the LEE in enterohemorrhagic *Escherichia coli*. PLoS One 7(9), e46288
Miyata ST, Unterweger D, Rudko SP, Pukatzki S (2013) Dual expression profile of type VI secretion system immunity genes protects pandemic *Vibrio cholerae*. PLoS Pathog 9(12), e1003752
Monjaras Feria J, Garcia-Gomez E, Espinosa N, Minamino T, Namba K, Gonzalez-Pedrajo B (2012) Role of EscP (Orf16) in injectisome biogenesis and regulation of type III protein secretion in enteropathogenic *Escherichia coli*. J Bacteriol 194(22):6029–6045
Navarro-Garcia F, Sonnested M, Teter K (2010) Host-toxin interactions involving EspC and pet, two serine protease autotransporters of the *Enterobacteriaceae*. Toxins 2(5):1134–1147
Navarro-Garcia F, Serapio-Palacios A, Ugalde-Silva P, Tapia-Pastrana G, Chavez-Duenas L (2013) Actin cytoskeleton manipulation by effector proteins secreted by diarrheagenic *Escherichia coli* pathotypes. BioMed Res Int 2013:22
Neves BC, Mundy R, Petrovska L, Dougan G, Knutton S, Frankel G (2003) CesD2 of enteropathogenic *Escherichia coli* is a second chaperone for the type III secretion translocator protein EspD. Infect Immun 71(4):2130–2141
Njoroge JW, Gruber C, Sperandio V (2013) The interacting Cra and KdpE regulators are involved in the expression of multiple virulence factors in enterohemorrhagic *Escherichia coli*. J Bacteriol 195(11):2499–2508
Nougayrede JP, Fernandes PJ, Donnenberg MS (2003) Adhesion of enteropathogenic *Escherichia coli* to host cells. Cell Microbiol 5(6):359–372
Oberhettinger P, Leo JC, Linke D, Autenrieth IB, Schutz MS (2015) The inverse autotransporter intimin exports its passenger domain via a hairpin intermediate. J Biol Chem 290(3):1837–1849
Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C (2005) Escape of intracellular *Shigella* from autophagy. Science 307(5710):727–731
Parsot C (2009) *Shigella* type III secretion effectors: how, where, when, for what purposes? Curr Opin Microbiol 12(1):110–116

Paton AW, Srimanote P, Woodrow MC, Paton JC (2001) Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. Infect Immun 69(11):6999–7009

Pearson JS, Giogha C, Ong SY, Kennedy CL, Kelly M, Robinson KS, Lung TW, Mansell A, Riedmaier P, Oates CV, Zaid A, Muhlen S, Crepin VF, Marches O, Ang CS, Williamson NA, O'Reilly LA, Bankovacki A, Nachbur U, Infusini G, Webb AI, Silke J, Strasser A, Frankel G, Hartland EL (2013) A type III effector antagonizes death receptor signalling during bacterial gut infection. Nature 501(7466):247–251

Pohlner J, Halter R, Beyreuther K, Meyer TF (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature 325(6103):458–462

Portaliou AG, Tsolis KC, Loos MS, Zorzini V, Economou A (2016) Type III secretion: building and operating a remarkable nanomachine. Trends Biochem Sci 41(2):175–189

Quitard S, Dean P, Maresca M, Kenny B (2006) The enteropathogenic *Escherichia coli* EspF effector molecule inhibits PI-3 kinase-mediated uptake independently of mitochondrial targeting. Cell Microbiol 8(6):972–981

Radics J, Konigsmaier L, Marlovits TC (2014) Structure of a pathogenic type 3 secretion system in action. Nat Struct Mol Biol 21(1):82–87

Reichow SL, Korotkov KV, Hol WG, Gonen T (2010) Structure of the cholera toxin secretion channel in its closed state. Nat Struct Mol Biol 17(10):1226–1232

Robinson KS, Mousnier A, Hemrajani C, Fairweather N, Berger CN, Frankel G (2010) The enteropathogenic *Escherichia coli* effector NleH inhibits apoptosis induced by *Clostridium difficile* toxin B. Microbiology 156(Pt 6):1815–1823

Roggenkamp A, Ackermann N, Jacobi CA, Truelzsch K, Hoffmann H, Heesemann J (2003) Molecular analysis of transport and oligomerization of the *Yersinia enterocolitica* adhesin YadA. J Bacteriol 185(13):3735–3744

Roman-Hernandez G, Peterson JH, Bernstein HD (2014) Reconstitution of bacterial autotransporter assembly using purified components. Elife 3, e04234

Romero S, Grompone G, Carayol N, Mounier J, Guadagnini S, Prevost MC, Sansonetti PJ, Van Nhieu GT (2011) ATP-mediated Erk1/2 activation stimulates bacterial capture by filopodia, which precedes *Shigella* invasion of epithelial cells. Cell Host Microbe 9(6):508–519

Rosenshine I, Ruschkowski S, Finlay BB (1996) Expression of attaching/effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. Infect Immun 64(3):966–973

Rudel T, Kepp O, Kozjak-Pavlovic V (2010) Interactions between bacterial pathogens and mitochondrial cell death pathways. Nat Rev Microbiol 8(10):693–705

Ruiz N, Kahne D, Silhavy TJ (2006) Advances in understanding bacterial outer-membrane biogenesis. Nat Rev Microbiol 4(1):57–66

Ruiz-Perez F, Nataro JP (2014) Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence. Cell Mol Life Sci 71(5):745–770

Ruiz-Perez F, Henderson IR, Nataro JP (2010) Interaction of FkpA, a peptidyl-prolyl cis/trans isomerase with EspP autotransporter protein. Gut Microbes 1(5):339–344

Ruiz-Perez F, Wahid R, Faherty CS, Kolappaswamy K, Rodriguez L, Santiago A, Murphy E, Cross A, Sztein MB, Nataro JP (2011) Serine protease autotransporters from *Shigella flexneri* and pathogenic *Escherichia coli* target a broad range of leukocyte glycoproteins. Proc Natl Acad Sci U S A 108(31):12881–12886

Sahl JW, Steinsland H, Redman JC, Angiuoli SV, Nataro JP, Sommerfelt H, Rasko DA (2011) A comparative genomic analysis of diverse clonal types of enterotoxigenic *Escherichia coli* reveals pathovar-specific conservation. Infect Immun 79(2):950–960

Salacha R, Kovacic F, Brochier-Armanet C, Wilhelm S, Tommassen J, Filloux A, Voulhoux R, Bleves S (2010) The *Pseudomonas aeruginosa* patatin-like protein PlpD is the archetype of a novel Type V secretion system. Environ Microbiol 12(6):1498–1512

Samba-Louaka A, Nougayrede JP, Watrin C, Oswald E, Taieb F (2009) The enteropathogenic *Escherichia coli* effector Cif induces delayed apoptosis in epithelial cells. Infect Immun 77(12):5471–5477

Sanada T, Kim M, Mimuro H, Suzuki M, Ogawa M, Oyama A, Ashida H, Kobayashi T, Koyama T, Nagai S, Shibata Y, Gohda J, Inoue J, Mizushima T, Sasakawa C (2012) The *Shigella flexneri* effector OspI deamidates UBC13 to dampen the inflammatory response. Nature 483(7391):623–626

Sanchez-Pulido L, Devos D, Genevrois S, Vicente M, Valencia A (2003) POTRA: a conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. Trends Biochem Sci 28(10):523–526

Sanchez-Villamil J, Navarro-Garcia F (2015) Role of virulence factors on host inflammatory response induced by diarrheagenic *Escherichia coli* pathotypes. Future Microbiol 10(6):1009–1033

Schmidt MA (2010) LEEways: tales of EPEC, ATEC and EHEC. Cell Microbiol 12(11): 1544–1552

Shen S, Mascarenhas M, Morgan R, Rahn K, Karmali MA (2005) Identification of four fimbria-encoding genomic islands that are highly specific for verocytotoxin-producing *Escherichia coli* serotype O157 strains. J Clin Microbiol 43(8):3840–3850

Shneider MM, Buth SA, Ho BT, Basler M, Mekalanos JJ, Leiman PG (2013) PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. Nature 500:350–353

Strasser A, Jost PJ, Nagata S (2009) The many roles of FAS receptor signaling in the immune system. Immunity 30(2):180–192

Tapia-Pastrana G, Chavez-Duenas L, Lanz-Mendoza H, Teter K, Navarro-Garcia F (2012) VirK is a periplasmic protein required for efficient secretion of plasmid-encoded toxin from enteroaggregative *Escherichia coli*. Infect Immun 80(7):2276–2285

Totsika M, Wells TJ, Beloin C, Valle J, Allsopp LP, King NP, Ghigo JM, Schembri MA (2012) Molecular characterization of the EhaG and UpaG trimeric autotransporter proteins from pathogenic *Escherichia coli*. Appl Environ Microbiol 78(7):2179–2189

Uhlen P, Laestadius A, Jahnukainen T, Soderblom T, Backhed F, Celsi G, Brismar H, Normark S, Aperia A, Richter-Dahlfors A (2000) Alpha-haemolysin of uropathogenic *E. coli* induces Ca2+ oscillations in renal epithelial cells. Nature 405(6787):694–697

Ulrich T, Oberhettinger P, Schutz M, Holzer K, Ramms AS, Linke D, Autenrieth IB, Rapaport D (2014) Evolutionary conservation in biogenesis of beta-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein. J Biol Chem 289(43): 29457–29470

Valle J, Mabbett AN, Ulett GC, Toledo-Arana A, Wecker K, Totsika M, Schembri MA, Ghigo JM, Beloin C (2008) UpaG, a new member of the trimeric autotransporter family of adhesins in uropathogenic *Escherichia coli*. J Bacteriol 190(12):4147–4161

Viswanathan VK, Lukic S, Koutsouris A, Miao R, Muza MM, Hecht G (2004) Cytokeratin 18 interacts with the enteropathogenic *Escherichia coli* secreted protein F (EspF) and is redistributed after infection. Cell Microbiol 6(10):987–997

Weiner JH, Li L (2008) Proteome of the *Escherichia coli* envelope and technological challenges in membrane proteome analysis. Biochim Biophys Acta 1778(9):1698–1713

Weisswange I, Newsome TP, Schleich S, Way M (2009) The rate of N-WASP exchange limits the extent of ARP2/3-complex-dependent actin-based motility. Nature 458(7234):87–91

Yang K, Meng J, Huang YC, Ye LH, Li GJ, Huang J, Chen HM (2014) The role of the QseC quorum-sensing sensor kinase in epinephrine-enhanced motility and biofilm formation by *Escherichia coli*. Cell Biochem Biophys 70(1):391–398

Yao Q, Lu Q, Wan X, Song F, Xu Y, Hu M, Zamyatina A, Liu X, Huang N, Zhu P, Shao F (2014) A structural mechanism for bacterial autotransporter glycosylation by a dodecameric heptosyltransferase family. Elife 3

Zhou Y, Tao J, Yu H, Ni J, Zeng L, Teng Q, Kim KS, Zhao GP, Guo X, Yao Y (2012) Hcp family proteins secreted via the type VI secretion system coordinately regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. Infect Immun 80(3):1243–1251

Zoued A, Durand E, Bebeacua C, Brunet YR, Douzi B, Cambillau C, Cascales E, Journet L (2013) TssK is a trimeric cytoplasmic protein interacting with components of both phage-like and membrane anchoring complexes of the type VI secretion system. J Biol Chem 288:27031–27041

Zoued A, Durand E, Brunet YR, Spinelli S, Douzi B, Guzzo M, Flaugnatti N, Legrand P, Journet L, Fronzes R, Mignot T, Cambillau C, Cascales E (2016) Priming and polymerization of a bacterial contractile tail structure. Nature 531(7592):59–63

Chapter 11
Therapeutics and Vaccines Against Pathogenic *Escherichia coli*

Marina S. Palermo, José Flores-Figueroa, and Mercedes Paredes-Paredes

Summary Diarrheagenic *E. coli* continues to be an important pathogen causing significant illness in animals and humans. Despite the fact that considerable investigations have been done following the initial discovery of enterotoxigenic *Escherichia coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC), there are currently no available licensed and broadly protective, ETEC nor EHEC vaccines. The significant, yet unsuccessful efforts in obtaining a vaccine candidate have provided important information on strategies to consider in future formulations. There are now new promising vaccine candidates based on this pioneering work, even though they have not generated successful vaccines. This chapter is focused on outstanding findings and their impact in human public health.

M.S. Palermo (✉)
Instituto de Medicina Experimental (IMEX)-CONICET—Academia Nacional de Medicina, Pacheco de Melo 3081, Buenos Aires C1425AUM, Argentina
e-mail: marinaspalermo@hotmail.com

J. Flores-Figueroa • M. Paredes-Paredes
JM Research SC, Avenida Teopanzolco 217, suite 2, Cuernavaca, 62290 Morelos, Mexico
e-mail: jose@jmresearch.org; mercedes@jmresearch.org

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_11

1 Recent Advances in *E. coli* Vaccines

1.1 Enterotoxigenic Escherichia coli (ETEC) Vaccines

1.1.1 Cellular Vaccines

ACE527 and ETVAX

The first cellular enterotoxigenic *E. coli* (ETEC) vaccine produced was the colicin E2-inactivated ETEC derived from the prototype strain H10407 (O78:H11, LT+ STa+ CFA/I+). Adult volunteers inoculated orally with this killed whole-cell product developed IgA antibodies to CFA/I fimbrial adhesin and heat-labile toxin (LT). As a result, volunteers were protected against a homologous challenge (Evans et al. 1988). Unfortunately, the anti-CFA/I and anti-LT antibodies produced were only effective against the homologous strain. Nonetheless, studies of this killed whole-cell vaccine led to the realization that an effective ETEC vaccine should minimally induce antibodies that protect against multiple CFs (colonization factors), adhesins, and LT (Zhang and Sack 2015).

The most widely studied whole-cell ETEC vaccine candidates are ETVAX and ACE527 (Bourgeois et al. 2016). The ACE527 vaccine is composed of three live attenuated *E. coli* strains with deletion mutations introduced at *aroC*, *ompC*, *ompF*. These genes collectively express five CFs adhesins (CFA/I, CS2, CS3, CS5, CS6), one CFA subunit (CS1), and the LTB subunit (Turner et al. 2001). In a phase I study, the oral inoculation containing live vaccine induced antibody responses to LTB, CFA/I, CS3, and CS6 among adults, but only reduced the severity of diarrhea following a homologous challenge (Harro et al. 2011). In a phase II b clinical trial with 70 adult volunteers, humoral response was observed against LT-B, CFA/I, and CS3; however, humoral response against CS6 was low. Protective efficacy was not significant and the number of mild and severe diarrhea cases after the challenge with ETEC strain H10407 was not reduced; nevertheless, this candidate reduced fecal shedding of H10407 on day 2 postchallenge (Darsley et al. 2012). Furthermore, a new ACE527 vaccine generation was designed in combination with a novel adjuvant dmLT (double-mutant LT) (Table 11.1). During a phase IIb challenge, the study not only showed 58.5 % efficacy in protecting against diarrhea of any severity, but also demonstrated protection against severe ETEC diarrhea with protective efficacy of 65.9 % (Bourgeois et al. 2016).

ETVAX is the most advanced, fully formulated, and completed vaccine ready for field testing according to the vaccine field experts (Bourgeois et al. 2016). The newest generation of ETVAX is based on Svennerholm and Holmgren's work. The ETVAX vaccine was first designed at the University of Gothenburg in Sweden. This vaccine includes a recombinant purified CT-B (Cholera-like toxin B subunit) and five different formalin-killed ETEC strains carrying seven CFs: CFA/I, CS1, CS2, CS3, CS4, CS5, and CS6 (Table 11.1). Despite carriage of CS6 by two of the strains, immunogenicity against this CF was not induced due to the effects of formalin treatment destroying the nonfimbrial structures. Two of the strains were originally

Table 11.1 Vaccines against enterotoxigenic *E. coli*

ETEC vaccine	Administration	Target	Status	Comment	References
Dukoral®	Oral	CB	Worldwide licensed	Licensed to prevent cholera expected cross-protection for LT	Clemens et al. (1988)
LT patch	Skin patch	LT	Phase III	Safe and immunogenic but limited protection	Behrens et al. (2014)
ACE527/dmLT (second generation vaccine)	Oral	CFA/I, LT	Phase II b	Protection efficacy 58.5 % against ETEC diarrhea of any severity. Protective efficacy of 65.9 % for severe ETEC diarrhea.	Darsley et al. (2015)
ETVAX (second-generation vaccine)	Oral	CT-B, ST, CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6	Phase II b	First generation showed significant protection against severe form of TD but not protective in a phase III pediatric trial	Bourgeois et al. (2016), and Sack et al. (2007)
ETVAX (latest generation with and without dmLT)	Oral	CT-B, ST, LT	Phase I/II	Currently being tested in Bangladesh with volunteer adults and children	https://clinicaltrials.gov NCT02531802
Hyperimmune bovine colostrum	Oral	Passive immunization	Phase II	90.9 % protection against diarrhea with the highest dose ($p=0.0005$)	Otto et al. (2011)
dmLT LTR192G/L211A	Vaccine adjuvant	LT	Phase II	Systemic and mucosal humoral immune response against LT were detected	El-Kamary et al. (2013)
CfaE-dmLT	Intradermal	LT	Phase I/IIb	Induced strong immune responses at systemic and mucosal sites	Harro et al. (2015)
Formalin-killed whole-cell mixture of five E. coli	Subcutaneous	EAEC, EPEC, EIEC, EHEC and ETEC	Preclinical	100 % survival rate among immunized mice compared to unimmunized controls ($p<0.001$)	Gohar et al. (2016)
STa toxoids (3XTaN_{12S}-dmLT toxoid fusion)	Intraperitoneal	ST and LT	Preclinical	Strong in vitro neutralization activity against STa in intraperitoneally immunized mice	Ruan et al. (2014a, b)
CFA MEFA (multiepitope fusion antigen)	in vitro	ETEC strains expressing the seven CFAs	Preclinical	The induced antibodies significantly blocked the in vitro adherence of *E. coli* strains expressing seven CFs	Ruan et al. (2014a, b)
CFAI/II/IV-3XSTa$_{N12S}$-dmLT MEFA	in vitro	LT and ST and ETEC strains expressing the seven CFAs	Preclinical	Induces equivalently protective antibodies against seven CFAs and both toxins	Ruan et al. (2015)

heat-stable toxin (ST) producers, but none of these expressed LT. During the efficacy evaluation of 1357 volunteers traveling from United States to Mexico or Guatemala, marginal reduction of the overall number of diarrhea cases attributed to ETEC strains was observed (Sack et al. 2007). Moreover, a phase III clinical trial showed little protection against ETEC diarrhea among very young children living in areas such as Egypt and Bangladesh, where ETEC diarrhea is endemic (Savarino et al. 2002; Svennerholm and Tobias 2008). In 2002, following the completion of the Phase III clinical trial in Egypt, the World Health Organization (WHO) reviewed the data from the Egyptian studies in the context of an international meeting held in Switzerland in 2003. During this time, participants reviewed progress in the ETEC vaccine development and were able to make recommendations for future directions in research. WHO recommended: to increase the amount of CF antigens delivered in each dose of the vaccine, to include CS6 in the vaccine formulation in order to improve coverage in the field, and to evaluate the potential value of adding a mucosal adjuvant to the formulation. In addition, WHO also suggested that the primary endpoint for future Phase III efficacy trials should be protected against severe disease and/or hospitalization associated with vaccine-preventable cases (WHO 2006).

To improve the efficacy of whole-cell vaccines, modifications that included the refinement of the volunteer challenge model, the overexpression of CFs, and the replacement of supplementary recombinant CTB with an LTB–CTB hybrid were implemented (Harro et al. 2011; Holmgren et al. 2013; Lundgren et al. 2013). The latest generation of ETVAX vaccine alone combined with dmLT as adjuvant is currently being tested in a Phase I/II clinical trial in Bangladesh with volunteer adults and children. Plans to complete this trial are scheduled on December 2016.

1.1.2 Passive Immunization

Hyperimmune Bovine Colostrum

Antibodies contained in hyperimmune colostrum confer passive immunization to infants of diverse mammal species. As a result, the University of Melbourne in Australia decided to further explore this approach. Extracts obtained from ETEC strains belonging to 14 different serogroups, commonly associated with diarrhea, and expressing CFs: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS12, CS14, and CS17, were injected into pregnant cows in 5 doses over a period of 10 weeks. Colostrum was obtained from the first milk after calving. Lyophilized colostrum mixed with adjuvant was orally administered to volunteers in different tablet formulations. This randomized, double-blind, placebo-controlled trial involved 90 healthy adult volunteers. The colostrum tablets conferred significant protection against diarrhea (90.9 % with the highest dose, 58.3 % with the lowest dose) after the challenge with the prototype ETEC H10407 strain (Otto et al. 2011). The group is presently exploring partners for further development.

1.1.3 ETEC Subunit Vaccine Candidates

Although whole-cell products rCTB-CF (or its modified product ETVAX) and ACE527 continue to improve in order to achieve better protective efficacy, subunit vaccines have shown to be promising and effective alternatives for the protection against ETEC diarrhea. In contrast to whole-cell products, subunit vaccines contain well-defined and highly characterized immunogens that induce immune-specific responses to those immunogens. Since subunit vaccines typically contain one or more well-characterized proteins or polypeptides, product safety can be more easily managed and achieved compared to whole-cell vaccines.

LT Patch

An early study revealed that mice transcutaneously immunized with an adhesive patch embedded with purified LT developed an antibody response to LT and accumulated less fluid in their intestines after an oral challenge with LT (Yu et al. 2002). When the patch was applied to humans (upper arm), immunized adults developed strong IgG and IgA antibody responses to LT (Güereña-Burgueño et al. 2002; Glenn et al. 2007). Even so, a subsequent placebo-controlled trial study, confirmed the induction of anti-LT antibodies, demonstrating that immunized subjects were only marginally protected against an ETEC challenge (McKenzie et al. 2007). Subsequently, a phase II clinical trial reported that transcutaneous immunization reduced the incidence of moderate-to-severe diarrhea caused by ETEC and other enteric pathogens by 75 % in healthy adults traveling to Mexico or Guatemala (Frech et al. 2008). This result led to the speculation that anti-LT immunity may provide broad-spectrum protection against a variety of enteric pathogens. Unfortunately, a phase III clinical trial failed to confirm results from the first small phase II trial. Data from the phase III clinical trial revealed that the LT patch provided 60 % protection against LT+ ETEC diarrhea, but failed to protect against STa+ ETEC or any other causes of diarrhea (Behrens et al. 2014). A later reanalysis of data from the first field trial found that protective efficacy was overestimated in the original publication (Ellingsworth 2011). Furthermore, a second phase III clinical trial with healthy adults traveling to India discovered that the LT patch alone was not protective against ETEC nor any diarrhea causes (Steffen et al. 2013). Consequently, the collective results of these studies, the safety concerns, and the use of the LT patch alone are no longer considered suitable approaches for vaccination against diarrhea attributable to ETEC (Riddle and Savarino 2014). The associated transdermal antigen delivery method, however, is innovative and worthy of further exploration as a means of delivering more promising immunogens with the goal of preventing ETEC or other bacterial diarrhea.

Mutant LT

Substantial inherent toxicity limits the utility of LT holotoxin as an orally administered vaccine. To overcome this, mutant forms of LT were designed to eliminate the toxic activity. Since, anti-LT antibodies have not demonstrated their broad efficacy in protecting against ETEC diarrhea, mutant LT alone has not been considered to be an effective immunogen for ETEC subunit vaccine development. Instead, it has been primarily explored as a vaccine adjuvant (Zhang and Sack 2015; Fleckenstein et al. 2014). A double mutant dmLT (LTR192G/L211A) was shown to lack toxicity in a mouse assay and exhibited greatly reduced activity in stimulating cyclic AMP levels in Caco-2 cells (Norton et al. 2011). Additionally, it appeared to be a safer antigen for inducing anti-LT antibody responses. In another toxin variant, two-point substitutions were introduced in order to disrupt ADP ribosylating activity, the toxic effect, and a putative pepsin cleavage site. Systemic and mucosal humoral immune responses against LT were detected, particularly in the group receiving 50 mg/dose. Thus, the dmLT was deemed a suitable adjuvant or candidate for inclusion in multiple-antigen formulations (El-Kamary et al. 2013).

STa Toxoids

It is well known now that a vaccine candidate carrying LT alone is not effective against ETEC diarrhea (Behrens et al. 2014; Steffen et al. 2013). We have also learned that moderate-to-severe diarrhea is most often attributed to STa+ ETEC strains. More than two-thirds of ETEC strains isolated from patients that developed diarrhea expressed STa alone or together with LT. However, ST toxoid-based vaccines faced inherent challenges that included the poor immunogenicity related to the small size of the toxin, and their similarity to endogenous human peptides guanylin and uroguanylin (Zhang and Sack 2015). The STa Consortium Group has been dedicated toward designing STa molecules that evade toxicity and that simultaneously elicit neutralizing antibodies that do not cross-react with the native guanylin and uroguanylin peptides.

Initially substituting a noncysteine amino acid residue into a full-length, porcine-type STa demonstrated a significant reduction in toxicity while the STa native protein structure was largely retained. This STa was later genetically fused to a single polypeptide carrying one copy of subunit A and one copy of subunit B from LT_{R192G} (monomeric LT mLTR192G). Eventually, this toxoid became immunogenic and antibodies induced by the resultant toxoid fusions neutralized both LT and ST (Zhang et al. 2010).

Toxoid STa_{P13F} (in which the 13th residue of proline was replaced with phenylalanine) was genetically fused to mLT_{R192G} (with STa_{P13F} at either the C or the N terminus, between A1 and A2, or between the A and B peptides of LT_{R192G}), to generate LT_{R192G}–ST_{P13F} toxoid fusion polypeptides. Immunization with these LT_{R192G}–ST_{P13F} fusion products induced anti-LT and anti-STa antibodies, which moderately neutralized STa in vitro.

In order to improve anti-STa immunogenicity, three copies of a different STa toxoid (STa_{A14Q}) were fused to a triple-mLT (tm LT), $LT_{S63K/R192G/L211A}$; STa_{A14Q} was fused to the N and C terminus and between the A1 and A2 peptides of the tm LT. This $3xSTa_{A14Q}$-tm LT fusion product exhibited enhanced anti-STa immunogenicity. For the first time, antibodies induced by this toxoid fusion completely neutralized purified STa in an in vitro antibody neutralization assay. The toxoid fusion $3XSTa_{A14Q}$-tm LT showed that it was safe and that it could induce neutralizing antitoxin antibodies in intraperitoneally immunized mice (Zhang et al. 2013).

A panel of 14 STa toxoids was selected from a constructed STa toxoid library to further optimize STa toxoids for an LT-STa toxoid fusion product that induced robust anti-STa antibody responses. Each of the 14 selected $STa_{toxoids}$ genetically fused three copies to a dmLT monomer to generate $3XSTa_{-toxoid}$–dmLT fusions. Of the 14 toxoid fusions examined, $3XSTa_{N12S}$–dmLT induced the highest anti-STa IgG titer and the third highest anti-STa IgA titer in intraperitoneally immunized mice, while antibodies induced by this product showed the strongest in vitro neutralization activity against STa (Ruan et al. 2014a, b). This $3XTaN_{12S}$–dmLT toxoid fusion is now the most promising target for antitoxin subunit vaccine development. Human volunteer studies will be conducted to further characterize antigen tolerance safety. Most importantly, studies will allow more protection against STa-producing ETEC infection (Zhang and Sack 2015).

Colonization Factors

There have been more than 25 unique CFs identified in the global collection of ETEC up to date from the initial identification of CFA/I. In addition, ongoing DNA sequencing projects suggest that new antigens will continue to be identified (Fleckenstein et al. 2014). There have been multiple approaches that incorporate the most prevalent CFs in candidate vaccines. Some of these approaches include those in whole-cell vaccines which are currently in the advanced stages of clinical development.

The U.S. Naval Medical Research Center (NMRC) has been exploring the use of a conservative CF adhesin to induce cross-protective antibodies against different CFs. It has been reported that some ETEC CFs, for example, CFA/I, CS4, CS14, CS1, CS17, and CS19, have homologous antigenic domains. The major subunits of these CFs, particularly the adhesive units or adhesins, have similar amino acid sequences (Gaastra et al. 2002). Moreover, their antibodies induced by one CF are able to cross-protect against antigenically related CFs (Zhang et al. 2015).

The adhesin tip from CFA/I, CfaE, has shown considerable promise for the development of a broadly protective ETEC antiadhesin vaccine. Mice and rabbits immunized transcutaneously with CfaE combined with LT, mLT, or dmLT (as an adjuvant) developed a robust anti-CFA/I antibody response that inhibited the attachment of CFA/I, CS2, CS4, CS14, and CS17 ETEC strains to bovine and chicken enterocytes. In a recent phase I study, a CfaE prototype vaccine induced strong

immune responses at systemic and mucosal sites (Steel et al. 2012; Laird et al. 2014). Another recently completed phase IIb immunization and challenge clinical trial with a CfaE-dmLT prototype vaccine also yielded encouraging results. These outcomes support the concept that, under appropriate conditions, a parenterally administered subunit vaccine may prevent ETEC diarrhea. Based on these encouraging phase I/IIb results, NMRC is now working with other partners to further accelerate the development of this concept as a complete vaccine. It is also optimizing the formulation, dose, adjuvant(s), and route of delivery to further improve and broaden vaccine-induced protection (Harro et al. 2015).

Epitope-Based Antigens

Advantages of epitope- or peptide-based antigens include product safety, ease of production, and most importantly, precise targeting of host immune responses. A major disadvantage, however, is the generally poor immunogenicity associated with the use of a single low-molecular-weight peptide or immunogen.

In order to develop this approach, a strongly immunogenic major subunit of CFA/I (CfaB) was used as a backbone (or a carrier protein). An epitope prediction software was utilized to retain the most important B-cell epitopes of CfaB. Then, surface exposed, but less immunogenic epitopes of CfaB were placed with the most immunogenic B-cell epitopes from other clinically important ETEC CFs, including CFA/II (CS1, CS2, CS3) and CFA/IV (CS4, CS5, CS6). The end product was a CFA multiepitope fusion antigen (MEFA) (Ruan et al. 2014a, b). This product was strongly immunogenic, as it induced high titers of antibodies specific to all seven of these CFAs and probably to other CFAs homologous to CFA/I. More importantly, the induced antibodies significantly blocked the in vitro adherence of *E. coli* strains expressing these seven CFs. Thus, this CFA MEFA represents a strong candidate for antiadhesin subunit vaccine development (Zhang and Sack 2015). This formulation and its administration regimen has been suggested to be potentially useful in the prevention of travelers' diarrhea.

Toxoid–CFA Fusions

Although the CFA MEFA induced protective antibodies against the seven most important CFAs, these antibodies would not be expected to protect against the remaining ETEC diarrhea cases. On the other hand, LT and STa, alone or together, are expressed by ETEC strains causing 100 % of ETEC diarrhea cases. Under this reasoning Zhang's team decide to fuse human-type CFA MEFA to toxoid fusion 3xSTaN12S–dmLT and explored the application of CFA–toxoid MEFA in ETEC subunit vaccine development. Mice immunized intraperitoneally with CFA/I/II/IV-2XSTa$_{N12S}$dmLT, a single polypeptide carrying the CFA MEFA, two copies of STa$_{N12S}$, and the LT-A2 and LTB peptides, developed antibodies to all seven CFAs

and both toxins (Ruan et al. 2015). This product was even modified to carry three copies of STa_{N12S} for further enhancement of anti-STa immunogenicity. The new CFA/I/II/IV-3XSTa_{N12S}dmLT has been shown to possess stronger anti-STa immunogenicity, and antibodies induced by this fusion product showed greater neutralizing activity against STa. Although coadministration of the CFA MEFA and toxoid fusion 3XSTa_{N12S}–dmLT induces equivalently protective antibodies against seven CFAs and both toxins, the single CFA/I/II/IV-3XSTa_{N12S}-dmLTMEFA is more cost effective to manufacture and administer. This CFAI/II/IV-3XSTa_{N12S}–dmLT MEFA can potentially be considered the most promising antigen for ETEC subunit vaccine development (Zhang and Sack 2015).

1.1.4 Formalin-Killed Whole-Cell Mixture

This vaccine candidate is a formalin-killed, whole-cell mixture of enteroaggregative, enteropathogenic, enteroinvasive, enterohemorrhagic, and enterotoxigenic *E. coli* pathogroups. This combined vaccine candidate was aimed to be cost effective. Its immune efficacy was evaluated with adjuvant (with cholera toxin B subunit) and without adjuvant formulation. In the process, mice were challenged intraperitoneally with living *E. coli* pathogroups or the combination of the five *E. coli* pathogroups. Significant increase in specific antibodies (IgG) and significant survival rate was found (Gohar et al. 2016).

1.1.5 Novel Antigens with Potential Utility in ETEC Vaccines

Novel Plasmid-Encoded ETEC Antigens

EtpA Adhesin

EtpA is a 170 kDa glycoprotein, secreted by a two partner secretion system. It is required for efficient colonization of the small intestine of a murine model of infection, and also for both adhesion and toxin delivery to target epithelial cells in vitro (Fleckenstein et al. 2014; Roy et al. 2012). EtpA appears to function in a unique fashion by forming a molecular bridge between highly conserved regions of flagellin available at the tips of ETEC flagella and the host cell surface (Roy et al. 2009a, b). Antibodies directed at either EtpA or the conserved regions of flagellin inhibited toxin delivery in vitro (Roy et al. 2012) and prevented intestinal colonization of mice following gastrointestinal challenge with ETEC (Roy et al. 2009a, b).

Data emerging from a number of molecular epidemiological studies suggest that EtpA may be reasonably conserved among the ETEC pathotypes. EtpA has also been identified in a variety of strains from distinct phylogenetic groups (Sahl et al. 2011) and from different geographical areas (Del Canto et al. 2011) where *etpA* genes were found in more than 70 % of the strains examined.

EatA Protease

EatA is a member of the serine protease autotransporter family of virulence proteins. Recent studies show EatA to have two functions in ETEC. EatA degrades the EtpA adhesin, thereby modulating bacterial adhesion (Roy et al. 2011). It later degrades MUC2, the major mucin in the intestinal lumen. EtpA significantly enhances toxin delivery by promoting bacterial access to cell surface receptors. Early studies have demonstrated that antibodies against the secreted passenger domain of EatA protect against colonization in a murine model of infection and also prevent toxin delivery in vitro.

The significant homology of EatA and SepA, an autotransporter identified in *Shigella flexneri*, suggests that EatA and similar proteins could represent important targets in hybrid ETEC-*Shigella* vaccines. Similar molecules are also expressed by other diarrheagenic *E. coli* strains, including enteroaggregative *E. coli*, which is associated with more severe forms of infection (Boisen et al. 2012). Therefore, targeting these mucin-degrading enzymes could be useful in protecting against a number of important enteric pathogens (Fleckenstein et al. 2014).

1.1.6 Novel Chromosomally Encoded Antigens

While these molecules are not specific to the ETEC pathogroup, they do appear to function in concert with pathogroup-specific molecules and could theoretically serve as putative targets for vaccine development.

YghJ Metalloprotease

YghJ is an effector molecule secreted by the type 2 secretion system (T2SS), which is also responsible for the secretion of LT. This molecule also appears to enhance delivery of LT, in part through its metalloprotease activity and degradation of MUC2, as well as its cell-surface bound MUC3. Antibodies against YghJ inhibit toxin delivery in vitro (Luo et al. 2014). Vaccination with YghJ has been shown to afford modest protection against extra-intestinal pathogenic *E. coli* (Moriel et al. 2010); however, it is not yet clear whether it offers protection against ETEC.

EaeH Adhesin

Molecular pathogenesis studies suggest that EaeH enhances ETEC interactions with intestinal epithelial cells and that it promotes toxin delivery in vitro, as well as mucosal colonization in vivo (Fleckenstein et al. 2014). Interestingly, vaccination with FdeC, an EaeH homolog, inhibited kidney colonization in mice infected with uropathogenic *E. coli* (Nesta et al. 2012); however, demonstration of protection against ETEC remains to be established.

Autotransporter Proteins

These proteins are either surface expressed or secreted. They are also often highly immunogenic and may serve as potential vaccine targets. Indeed, two chromosomally encoded proteins in ETEC, antigen 43, and pAT are highly immunogenic. These are recognized during convalescent immune responses to ETEC and are protective against ETEC in a small intestinal colonization murine model of infection (Harris et al. 2011). These proteins deserve additional consideration as potential vaccine targets.

Flagellin Proteins

Motility appears to be an absolute requirement for effective delivery of LT toxin to the epithelial surface (Fleckenstein et al. 2014). Flagellin (FliC) is the major protein subunit of flagella, with an estimated of 20,000 molecules per flagellum; therefore, FliC is by far the most abundant protein secreted by ETEC and is recognized during the course of infection. Antibodies against conserved regions of the flagellin protein appear to protect against ETEC colonization in vivo (Roy et al. 2009a, b) and also impair bacterial adhesion in vitro. These virulence features are thought to be important prerequisites for toxin delivery.

1.1.7 Plant-Based Vaccines

Conceptually, oral plant-based vaccines are ideal because the manufacturing process is simple. No additional medical devices are needed for injection, and both antigen immunogenicity and biological activities are preserved in the gastrointestinal tract due to their natural bioencapsulation in a plant cell organelle (Takeyama et al. 2015).

As bioreactors, plants may yield high amounts of recombinant proteins. Recombinant subunit vaccines are safer than traditional vaccines since they do not contain live pathogens (Takeyama et al. 2015). The heat-labile enterotoxin B (LTB) subunit from ETEC and the cholera toxin B (CTB) subunit from *V. cholerae* were expressed under the control of the rice (Oryza sativa) globulin (Glb) promoter. LTB and CTB proteins were detected in transgenic rice by Western blot analysis. Immunogenicity of rice-derived CTB and LTB antigens were evaluated through oral and intraperitoneal administration in mice, respectively. The results revealed that specific IgG levels were enhanced in the sera of intraperitoneally immunized mice. Similarly, the toxin-neutralizing activity of CTB and LTB in serum of orally immunized mice was associated with elevated levels of both IgG and IgA (Soh et al. 2015).

1.2 *Enterohemorrhagic E. coli (EHEC)/Shiga Toxin-Producing E. coli (STEC) Vaccines*

1.2.1 Vaccines Against EHEC/STEC for Animals

Prevalence of Shiga-toxin (Stx)-associated hemolytic uremic syndrome (HUS) is closely related to the incidence of STEC infections. Since cattle are the main reservoir of STEC pathogens, many efforts have been focused on reducing STEC animal carriage. Potential ways to minimize STEC animal carriage include animal vaccinations, additives, manipulation of animal feeds, as well as farming practices (Thomas and Elliott 2013). Two commercially available vaccines exist; the first is based on the *E. coli* O157:H7 siderophore receptor and porin protein (SRP), which presumably target bacterial iron uptake and have reduced cattle fecal shedding and transmission of STEC (Thomson et al. 2009; Wileman et al. 2011) (Epitopix LLC, Willmar, Minnesota, U.S.) (Table 11.2). Whereas the second is based on secreted protein preparations containing components of the bacterial type three secretion system (T3SS) proteins from *E. coli* O157, the most predominant serotype worldwide associated with disease (Allen et al. 2011) (Econiche, Bioniche Life Sciences Inc., Belleville, Ontario, Canada). Several studies have also proposed bovine vaccination with formulations based on TTSS proteins (Snedeker et al. 2012; Smith et al. 2008; Vilte et al. 2011; Zhang et al. 2012). Recently, inclusion of Tir or H7 flagellin together with TTSS proteins reduced shedding below 10^4 CFU/g feces (McNeilly et al. 2015). Some studies have shown that although Stx does not play a major role during STEC pathogenesis in cattle, a partial suppression of bovine mucosal immune response by Stx could hamper anti-STEC response. Thus, some protocols have included the Stx, as an additional antigen in the vaccine formulations for domestic ruminants (Zhang et al. 2012). A similar approach was followed for preventing the edema disease, which is induced after infection with Stx2e producing STEC strains in pigs (Ren et al. 2013; Rossi et al. 2013; Sato et al. 2013). All these protocols have reported a significant rise of specific antibodies, together with a reduction of both cattle fecal shedding and intestine colonization of *E. coli* O157:H7. However, the effectiveness to control contamination of reservoirs remains unsatisfactory. In addition to the aforementioned approaches, interventions based on animal feed manipulations have included the addition of probiotics (Callaway et al. 2009; Stanford et al. 2014) and grain versus forage feeds. Although some of these practices have significantly reduced fecal shedding of *E. coli* O157:H7 and have even been proposed to be used during preslaughter to reduce *E. coli* contamination, in a large-scale randomized controlled trial, no associations have been found between *E. coli* O157:H7 load and feeding dried distiller's grain (Thomas and Elliott 2013). On the other hand, reducing the wet distillers' grain concentration for 56 days prior to slaughter reduced the shedding of STEC. Similarly, the addition of chlorate to feed or water was effective in the reduction of *E. coli* O157:H7 populations in ruminant animals. Nonetheless, feed supplementation with vitamin D (Thomas and Elliott 2013) and other additives such as ionophores or growth promoters resulted in being ineffective to control cattle contamination.

Table 11.2 Vaccines against enterohemorrhagic *E. coli* (EHEC) and/or Shiga-toxin producing *E. coli* (STEC)

Vaccine/disease	Antigen	Status/study	Comment	References
Animal vaccines				
Econiche, Bioniche Life Sciences Inc., Belleville, Ontario, Canada	TTSS proteins from *E. coli* O157. Interfere with EHEC adherence and colonization.	Commercially available	It reduces the fecal *E. coli* O157 carrier status. The effectiveness to control contamination of reservoir is still unsatisfactory	Allen et al. (2011)
Epitopix LLC, Willmar, Minnesota, U.S.	SRP protein	Commercially available	It interferes with iron uptake. For use in cattle ≥ 5 months of age to reduce *E. coli* O157 carrier status	Thomson et al. (2009)
EHEC carrier status in cattle	TTSS proteins (EspB, γ-intimin)	Experimental protocol in cattle	It reduces the amount of *E. coli* O157 shed in feces	Vilte et al. (2011)
EHEC carrier status in cattle	Combinations of TTSS proteins (Esp A, Intimin, Tir) and/or H7 flagellin	Experimental protocol in cattle	It reduces the amount of *E. coli* O157 shed in feces	McNeilly et al. (2015)
Porcine edema disease	mStx2e and fusion proteins: Stx2eA2B-His and Stx2eB-His	Experimental protocol in adult mice	Only mStx2e was effective to rise Abs and to confer protection against 1LD of Stx2e	Sato et al. (2013)
Porcine edema disease	Fusion protein: FedA subunit of the F18 fimbriae and Stx2eB	Experimental protocol in adult mice	Mice immunized with transgenic tobacco seeds via the oral route showed increase in IgA-positive plasma cells in the intestine	Rossi et al. (2013)
Porcine edema disease	DNA vaccine encoding Stx2eB and FedF	Experimental protocol in adult mice	Coimmunization with Stx2eB and FedF is effective for protecting mice from a challenge with *E. coli* O139	Ren et al. (2013)
EHEC carrier status in goats	Stx2B-Tir-Stx1B-Zot fusion protein	Experimental protocol in goats	Immunized 3-month-old goats showed less shedding of *E coli* O157:H7 after challenge	Zhang et al. (2012)
Human vaccines				
E. coli O157 human infection	O157-specific LPS coupled to rEPA	Phase I clinical trial, involving adult humans	The vaccine is safe and highly immunogenic	Ahmed et al. (2006)
E. coli O157 human infection	rEPA	Phase II clinical trial, involving fifty 2- to 5-year-old children	The vaccine triggers humoral IgG response with bactericidal activity	Ahmed et al. (2006)

(continued)

Table 11.2 (continued)

Vaccine/disease	Antigen	Status/study	Comment	References
E. coli O157 human infection	rEPA and Stx1-B subunit	Preclinical studies with weaning mice	Bactericidal Abs against *E. coli* O157, and neutralizing Abs against Stx1, but no cross-neutralization against Stx2	Szu and Ahmed (2014)
Stx-dependent systemic complications[a]	Stx2B-His plus a mLT	Preclinical studies with adult mice	Serum and lung neutralizing Abs. Anti-Stx2B Abs cross-neutralized Stx1. 50 % survival against 1LD of Stx1 or Stx2	Tsuji et al. (2008)
Stx-dependent systemic complications[a]	Chimeric protein between Stx2B and BLS	Preclinical studies with adult and weaning mice	Serum neutralizing Abs against Stx2a, Stx2c, Stx2d, and Stx1. 100 % survival against Stx2 challenge and EHEC oral infection	Mejías et al. (2013)
Stx-dependent systemic complications[a]	mOMVs from MsbB- and STxA-deficient EHEC O157:H7 bacteria	Preclinical studies with adult mice, without the use of commercial adjuvants	Humoral and mucosal protective immune responses after challenge with a lethal dose of wtOMVs	Choi et al. (2014)
Stx-dependent systemic complications[a]	Stx1B and mStx2A	Experimental protocol with adult mice	Neutralizing anti-Stx2/Stx1 Abs and 93 % survival against challenge of lysed *E. coli* O157:H7	Cai et al. (2011)
Stx-dependent systemic complications[a]	EspA, intimin, and Stx2B	Experimental protocol with adult mice	Protection against live EHEC or EHEC lysates. Induced humoral response and clearance of intestinal bacteria	Gu et al. (2009)
Stx-dependent systemic complications[a]	Stx2B/Stx1B/Intimin	Experimental protocol with adult mice	High titers of anti-Stx1 and Stx2 Abs, and protection against lethal infection with EHEC	Gao et al. (2011)
STEC infections and Stx-dependent systemic complications	Nonacetylated PNAG conjugated to Stx1B	Passive immunization in 5-day-old mice	Serum with bactericidal activity in vitro and effective against 6 STEC serogroups, including *E. coli* O157:H7	Lu et al. (2014)

TTSS type three secreted system, *SRP* siderophore receptor and porin, *Esp E. coli* secreted protein, *StxB* B subunit of Stx, *StxA* A subunit of Stx, *mStx* nontoxic mutant of Stx holotoxin, *mLT* mutant heat-labile enterotoxin, *LPS* lipopolysaccharide, *FedF* adhesin of the F18 fimbriae, *rEPA* recombinant exo-protein A of *P. aeruginosa*, *Abs antibodies*, *BLS* enzyme lumazine synthase from *Brucella* spp, *mOMV* modified outer membrane vesicles, *wtOMV* wild-type OMV, *MsbB* lipid A acyltransferase activity, *PNAG* poly-*N*-acetyl glucosamine

[a]Secondary to STEC infections. There is no clinical study for Stx-based human vaccines

Another preventive measurement proposed as a means of controlling STEC in cattle has been the application of lytic phages. The lytic phages have been shown to reduce STEC numbers in vitro, in food products (Sillankorva et al. 2012), and in some ruminants (Sheng et al. 2006). Phage-containing products that can be sprayed on animal hides or on meat products to control STEC organisms are available on the market and are also approved by the Food and Drug Administration (FDA) (Sillankorva et al. 2012). However, the efficacy of oral treatment of cattle with lytic phages was reported to be limited and to require the development of an enhanced approach for the delivery mode. Finally, improved hygiene and farming practices, as well as the solarization of soil in feed-lot pens, should be included as regular practices (Berry and Wells 2012). All of these strategies, however, have reached moderate success.

1.2.2 Vaccines Against EHEC/STEC for Humans

Prevention of human STEC infection has been difficult because although the main infection route includes contaminated cattle-derived food products, such as beef, milk, or dairy, other cross-contaminated sources such as leafy green vegetables and fruits have also been reported. Other contamination sources have included nonfood products, such as pool or drinking water, and the petting of zoo animals. Moreover, in Latin America, particularly Argentina, where STEC infections are endemic, person-to-person transmission has also been identified as a very important route of infection (Rivas et al. 2014). Thus, control measures covering all these points are unlikely to be available in many of these countries. Based on this postulation, Szu et al. developed polysaccharide conjugate vaccines composed of detoxified LPS for *E. coli* O157, covalently linked to, a carrier protein and a recombinant exoprotein of *Pseudomonas aeruginosa* (rEPA). Phase I and phase II clinical studies were conducted in adults and in children ranging from 2 to 5 years old, respectively (Ahmed et al. 2006) (Table 11.2). The *E. coli* O157 conjugate vaccines were safe for all ages, and a positive humoral IgG response with bactericidal activity was found in both age populations. However, there were certain limitations for using LPS-based vaccines. For example, LPS failed to induce a long-lasting humoral immune response especially in children and STEC non-O157 serotypes were not covered. In addition, the induced LPS antibodies had bactericidal activity, which could increase the incidence of HUS through toxin released upon lysis of bacteria. In one attempt to compensate for this shortcoming, the same group conjugated O-specific polysaccharide OSP with the B subunit of Stx1 (Szu and Ahmed 2014). However, this formulation did not neutralize Stx2—the toxin type most frequently found in severe HUS cases.

1.2.3 Vaccines Against Stx for Humans

The common and central virulence factor in all STEC strains that is secreted by both the O157 and non-O157 serotypes is Stx; therefore, an ideal human vaccine is one that elicits neutralizing antibody against Stx. Although two major types (Stx1 and Stx2)

and several subtypes (variants) have been described, Stx2 and its variant Stx2c are the members most frequently associated with severe complications, such as HUS cases or neurological complications, among STEC-infected subjects (Friedrich et al. 2002). Subsequently, various Stx-based vaccine approaches have been attempted. Some authors have reported that Stx1 and Stx2 do not provide heterologous protection through their B subunits; probably because Stx1 and Stx2 differ significantly in amino acid sequences. Conversely, Stx2c and Stx2d variants are readily neutralized with antibodies against Stx2. Thus, the Stx2 B subunit or the nontoxic recombinant Stx2 mutants were included as part of the vaccine component (Gupta et al. 2011; Mejias et al. 2013; Sato et al. 2013; Tsuji et al. 2008). Still, some approaches included Stx1–Stx2 fusion proteins (Cai et al. 2011; Gao et al. 2011; Smith et al. 2006). Concurrent immunization with multiple antigens including pathogenic factors involved in colonization and Stx may generate synergistic protection. They may broaden the coverage to the non-O157 STEC serotypes, and may allow both, preventive and therapeutic treatments (Garcia-Angulo et al. 2013; Gao et al. 2011; Gu et al. 2009). Being that all tested clinical STEC strains expressed PNAG, a novel approach has raised the possibility to target STEC bacteria with an anti-Poly-N-acetyl glucosamine (PNAG) multispecie vaccine (Lu et al. 2014). Considering the importance of Stx to develop systemic complications, a vaccine consisting of a synthetic monomer of nonacetylated PNAG conjugated to the B subunit of Stx1 was produced. However, antibodies raised in mice neutralized Stx1 potently, but modestly Stx2. In this model, neither antibodies to Stx1 alone nor PNAG alone were protective; both became essential in conferring in vivo protection to infant mice challenged with strains either producing Stx1 or Stx2. Other interesting developments included attenuated bacterial cells (Fujii et al. 2012; Gu et al. 2011; Rojas et al. 2010) and bacterial envelope/membrane derivatives (Cai et al. 2010; Choi et al. 2014) in addition to DNA vaccines (Bentancor et al. 2009). Favorable results have been shown when these vaccine preparations were assessed in animal models (reviewed in Garcia-Angulo et al. 2013).

Another obstacle that human vaccine development encounters is the planning of a trial to demonstrate its effectiveness. Most STEC cases occur during outbreaks in no particular location or region. Due to the unpredictable nature of the disease epidemiology, designing an efficacy trial for future human vaccines bears inherent difficulty.

2 Recent Advances in *E. coli* Therapy

2.1 Treatment of Diarrheagenic E. coli Pathogroups

The initial treatment of patients suffering from diarrhea due to a diarrheagenic *E. coli* pathogroups should include rehydration, which can be accomplished with an oral glucose or starch-containing electrolyte solution in most of the cases. The WHO recommended that oral rehydration solutions be prepared by mixing 3.5 g of NaCl,

2.5 g of $NaHCO_3$ (or 2.9 g of Na citrate), 1.5 g of KCl, and 20 g of glucose or any other different glucose polymer (e.g., four tablespoons of sugar or 50 g of cooked cereal flour, such as rice, maize, sorghum, millet, wheat, or potato) per liter of water. This makes a solution of approximately 90 mM of Na, 20 mM of K, 80 mM of Cl, 30 mM of HCO_3, and 111 mM of glucose.

Travelers' diarrhea continues to be the most common complaint among people from developed nations when visiting highly endemic countries (Flores-Figueroa et al. 2011). This is especially apparent during the summer months (Paredes-Paredes et al. 2011a) and is mostly associated with ETEC (Flores et al. 2008). Antibiotic therapy for adults suffering from travelers' diarrhea is widely recommended. There are multiple options to choose from when deciding treatment. Although there is growing concern about the increasing rate of antibiotic resistance among *E. coli*, particularly the occurrence of extended spectrum beta lactamases (ESBL) (Franz et al. 2015), well-known antibiotics are still recommended as empirical treatment. The mainstay of treatment includes antibacterial therapy with one of three drugs: a fluoroquinolone, rifaximin (i.e., rifamycin), or azithromycin (Paredes-Paredes et al. 2011b). Since empirical treatment is preferred most of the time, special consideration should be made with epidemiologic information. Some authors recommend ciprofloxacin to be the standard treatment in self-therapy of traveler's diarrhea, except when patients are located in South or Southeast Asia, where azithromycin is preferred (Steffen et al. 2015). ETEC and EAEC have high rates of resistance against ampicillin, trimethoprim-sulfamethoxazole, and doxycycline worldwide. High resistance rates are also present against quinolones in India and other Asian countries (Ouyang-Latimer et al. 2011).

Newer antibiotics, such as rifaximin and rifamycin (DuPont et al. 2014) have demonstrated to be effective and have significantly less adverse effects as compared to the standard therapy (DuPont et al. 2007). Prophylaxis for travelers' diarrhea using antibiotics should be reserved to selected cases involving high risk and instances when self-treatment is not feasible since the offered protection is not complete (Flores et al. 2011).

2.2 Treatment of STEC-Associated Human Disease

STEC infections can cause illness with a wide spectrum of severity. These infections can range from watery diarrhea and hemorrhagic colitis to neurological damage and HUS (Palermo et al. 2009), the most life-threatening complication related to Stx production. Because the intestine is the primary site of STEC infection, one of the greatest challenges would be to prevent HUS development through effective anti-STEC treatment in the gut. However, there is currently no preventive treatment that can lessen the risk of serious clinical complications such as HUS or neurologic damage during the intestinal phase of disease (Palermo et al. 2009). However, a rapid and appropriate health response to the management of acute bloody diarrhea is a necessary step in preventing HUS. Children with positive STEC infections

should remain isolated to prevent spread of infection in schools or between childcare mates. Antidiarrhea and antimotility agents should be avoided, as prolonged intestinal retention of bacteria can augment toxin exposure (Palermo et al. 2009).

Clinical management of individuals with STEC/EHEC infection is usually limited to supportive interventions such as hydration. Novel means for limiting disease duration and/or pathogen dissemination could therefore be beneficial for both individual patients and affected communities (Ahn et al. 2008).

The classical antibiotic treatment used to eliminate *E. coli* pathogens from the gut has been contraindicated (Wong et al. 2012). At antimicrobial levels required to inhibit bacterial replication, several antibiotics such as quinolones, including ciprofloxacin, produce DNA damage. This induces the SOS response with the unwanted secondary effect of simultaneously triggering phage production and *stx2* gene expression (Hauswaldt et al. 2013). In other cases, treatment of STEC with a lethal dose of antibiotics may cause a large toxin bolus to be released during the same time bacteria die. This simultaneous release would result in a very detrimental outcome for patients. These facts support the epidemiological observation that treatment of STEC infections with these antibiotics increases the risk of developing HUS (Wong et al. 2012). In contrast, a recent study reported that antibiotics such as meropenem, azithromycin, rifaximin, and tigecycline did not influence the phage and toxin levels of *E. coli* O104:H4 strain in vitro (Bielaszewska et al. 2012). Furthermore, it has been recently reported that the ketolide telithromycin and the related fluorketolide solithromycin repressed T3SS at concentrations of the antibiotics at which the growth of the bacteria still occurred, although at slightly reduced rates. This report showed that telithromycin and solithromycin at subminimum inhibitory concentration did not induce SulA expression, used as a bioreporter for microbial cytotoxicity by different DNA damaging agents, thus suggesting that Stx production is not induced with these antibiotics (Fernandez-Brando et al. 2016). However, further studies in animal models, as well as careful analyses of clinical outcomes in patients treated with these antibiotics are necessary to conclude if this is a general or a particular recommendation.

Alternative treatments to fight STEC/EHEC intestinal infections by avoiding the adverse side effects of antibiotics have been the focus of intensive research. Among them, some researchers have displayed bacteriocins as a new weapon to direct their lytic ability toward certain specific bacterial strains. Notably, unlike the case with many antibiotics, pyocin-mediated cell killing did not induce the release of Stx. Recently, the in vivo efficacy of a novel pyocin variant which expresses a higher ability to interact with some *E. coli* O157:H7 strains that produced low levels of O antigen has been assayed in the infant rabbit model (Ritchie et al. 2011). This treatment showed advantages specifying the effectiveness for reducing pathogenic *E. coli* colonization and shedding even when it was applied after diarrhea had started. However, the reduction in the amount of bacteria in the gut does not warrant that the toxin is below the threshold required to induce systemic disease. In addition, the fact that this is a therapy specific for a serogroup, it would be pertinent only for STEC O157 infections.

Other strategies directed to bacteria included the use of molecules that target a conserved membrane histidine sensor kinase, QseC, used by the bacterium to respond

to host signals (Rasko et al. 2008). Thus, inhibiting QseC-mediated activation of virulence gene expression neither killed EHEC, nor triggered the EHEC SOS response. In contrast, it decreased the expression of the *stxAB* genes. However, in vivo administration of this drug to infant rabbits before, during, or after EHEC resulted in a statistically nonsignificant reduction in EHEC colonization of the intestine. The authors proposed that the absence of in vivo protective effect in this animal model may have been attributed to the rapid absorption from the gastrointestinal tract and a nonabsorbable formulation may be required for noninvasive human pathogens such as EHEC. Other groups have similarly demonstrated that the divalent cation zinc inhibits EHEC adherence to cultured cells. In addition, zinc inhibits the expression of EHEC secreted protein A (EspA) and Stx at both the protein and the RNA levels (Crane et al. 2011). Nevertheless, the effect of this cation on the prevention of STEC/EHEC disease in an in vivo model has not been demonstrated. Considering that TTSS components are expressed transiently and not constitutively, a recent study demonstrated the ability of two inhibitory peptides designed to interact with EspA, to block colon damage induced by *C. rodentium* in mice (Larzábal et al. 2013).

Several studies have proposed that probiotics may have a relevant utility in preventing or ameliorating the course of STEC/EHEC infections, because they are capable of disrupting host-infectious agent/toxin interactions. They do this by occupying cellular receptors themselves, by producing decoy receptors for toxins, or by modifying the local milieu. Experiments in vitro have shown evidence of the biological competence between probiotic organisms with STEC/EHEC strains in epithelial adhesion, bacterial growth, and Stx2 secretion (Rund et al. 2013). For example, competence in one in vitro study showed that cultivation of EHEC organisms in the presence of various *Bifidobacterium*, *Pediococcus*, and *Lactobacillus* strains resulted in a decreased production of Stx2. This was attributed to a decrease in pH due to the acids produced by these agents (Carey et al. 2008).

Moreover, in different animal models, it was shown that the administration of probiotics prior to an experimental EHEC infection reduces pathology, by preventing intestinal colonization and damage, by bacterial translocation, and by Stx absorption (Chen et al. 2013). The extent of probiotic protective capacity seen in experimental models is likely dependent on the probiotic strain used and its ability to modify the intestinal medium. For example, the production of butyric acid and lactic acid by the probiotic agent has been revealed to be an important factor to inhibit EHEC strains.

2.2.1 Specific Anti-Stx Treatments

Stx are the key STEC virulence factors that lead to HUS development. Therefore, agents that interfere with Stx toxin binding, cellular uptake, trafficking, or RNA N-glycosidase activity are suitable candidates for specific treatment that prevent systemic complications derived from STEC infections.

Early approaches were based on the capture of free Stx in the gut via oral administration of either amorphous compounds or synthetic polymers (Watanabe-Takahashi et al. 2010). For example, Synsorb Pk was evaluated in human trials with

great expectation, but it was found not to be beneficial in preventing extra-renal complications and failed to decrease the duration of dialysis in children with new-onset of HUS (Trachtman et al. 2003).

Following the same rationale, other proposals encouraged the use of nonpathogenic recombinant bacteria expressing a Stx receptor mimic on its surface (Hostetter et al. 2014). Because the involvement of a genetically modified organism posed a barrier to clinical development, the same group has recently developed Gb3 receptor mimic bacterial ghosts, which are able to bind significant amounts of Stx1 and Stx2 in vitro, and that are highly efficacious against STEC challenge (Paton et al. 2015).

As a novel alternative treatment, a globotriose–chitosan conjugate was developed. After oral administration, the conjugate demonstrated it could prevent toxin entry into the bloodstream, enabling the mice to resist a fatal STEC challenge (Li et al. 2012).

The major drawback of all these proposals has been that barely traces of Stx that reach circulation would be enough to induce HUS, although these compounds show a high affinity for Stx and can neutralize significant amounts of Stx in the intestine.

Taking this limitation into account, several synthetic polymers with high avidity for Stx were designed to be used systemically (Mulvey et al. 2003; Nishikawa et al. 2005). These multivalent carbohydrate compounds neutralized Stx in vitro and were demonstrated to protect challenged mice when administered intravenously. Preparations of monoclonal antibodies (mAbs) that are able to bind Shiga toxins as well as their neutralizing effects in vitro and throughout different animal models have been reported (Cheng et al. 2013; Melton-Celsa and O'Brien 2013; Tzipori et al. 2004). Some of these mAbs have been humanized and evaluated in healthy volunteers during phase I studies (Bitzan et al. 2009; Dowling et al. 2005). In addition, a phase II study with chimeric mAbs against Stx1 and Stx2 is currently taking place in South America, but there is still no conclusive evidence regarding their therapeutic efficacy (López et al. 2010, 2015; Taylor et al. 2011). Although much of these mAbs recognize the B subunit of Stx to avoid the interaction of Stx with the Gb3 receptor, Tzipori's group generated an anti-Stx2 directed toward the A subunit. This was able to alter Stx2 intracellular trafficking and allowed the protection of piglets and mice from STEC challenge (Akiyoshi et al. 2010).

In addition to conventional antibodies, members of the camelid family also produced unusual antibodies that were solely composed of heavy chains. The antigen-binding site of these antibodies was composed of one variable heavy domain (VHH). Since single domain camelid antibodies are prone to recognize conformational epitopes into protein crevices, they would be ideal for the neutralization of the Gb3-binding activity of Stx2. VHH-neutralizing agents (VNAs) consist of linked VHHs that bind and neutralize Stx1 and Stx2. They proved to be protective in vivo when they were administered together with an "effector" conventional antibody (Tremblay et al. 2013). Recently, the possibility of gene delivery through a nonreplicating recombinant adenovirus has been shown to induce in vivo expression of the therapeutic VNAs. Only one intramuscular injection of the adenovirus vector carrying a secretory transgene of VNA-Stx protected the mice challenged

with Stx2. It also protected gnotobiotic piglets infected with EHEC from fatal systemic intoxication when it was given to piglets, 24 h or 48 h after bacterial challenge (Sheoran et al. 2015).

In addition, two copies of a Stx2B-binding VHH with high in vitro neutralizing activity (subnanomolar range) obtained after llama immunization with a novel Stx2B-based immunogen were fused to one antihuman seroalbumin VHH (Mejias et al. 2016). This engineered antibody showed increased permanence in circulation (15 days) and was able to neutralize the in vivo effects of Stx2 in three different mouse models of Stx2 toxicity and EHEC oral infections. In contrast to the previous VNAs, the trivalent molecule did not require a conventional mAb to be highly protective in vivo. This led to two advantageous consequences: (1) it avoided Fc-dependent cellular interactions and subsequently undesired side effects derived from these interactions and, (2) the commercial development is now more simple and less expensive compared to the strategy requiring the addition of a conventional mAb. This fact may have been related to the quality of the monomeric VHHs, underlining the importance in the selection of the immunogen during the development of protective antibodies.

2.2.2 Treatments That Interfere with Cellular Response to Stx

Although these approaches have been only demonstrated in animal models, during recent years, new therapeutic strategies have been developed to effectively hamper Stx-dependent tissue damage. A novel therapeutic strategy was based on short-term inhibition of host Gb3 synthesis to reduce binding and uptake of Stx by host cells. In this regard, C-9 (Genzyme Corp, USA), a specific inhibitor of glucosylceramide synthase, decreased Gb3 expression levels and prevented the cytotoxic effects of Stx2 on primary cultures of human renal epithelial and endothelial cells. The protective efficacy of C-9 was also shown in an experimental HUS rat model (Silberstein et al. 2011), suggesting that the inhibition of Gb3 synthesis may be a potential treatment for protection against the Stx toxic effects.

Furthermore, other findings have shown that inhibitors of Stx-mediated MAPK response like DHP-2 may prevent the Stx-induced inflammation that is thought to contribute to pathogenesis by STEC. Recently, the pretreatment of infant rabbits with the drug Imatinib, a MAPK inhibitor, resulted in a decrease of Stx-mediated heterophil infiltration, suggesting that it may act as potential therapeutic target for treating Stx-associated illnesses (Stone et al. 2012). In addition, another recent study indicated that manganese interfered with the retrograde movement of Stx to the Golgi, leading to increased degradation of the toxin in lysosomes. Pretreatment of mice with this metal reduced the lethal effects of Stx, raising a promise for a future clinical use (Mukhopadhyay and Linstedt 2012). As a general protector against apoptosis, ouabain was demonstrated to protect rat renal proximal tubules from Stx-induced cellular death (Burlaka et al. 2013). Although kidneys from ouabain-treated mice showed reduced podocyte depletion as compared to controls after Stx2 intoxication, the impact of this cellular protection on the final Stx2-dependent lethality was not reported.

Although progress in the discovery and development of drugs to block the action of the Stx appears as the most potent and promising treatment to ameliorate HUS, the very small window for its application remains as the current limitation for its success. In particular, the diagnosis of STEC infection has to be made within 2 days following the initiation of diarrhea. This is a challenge that cannot be routinely achieved, particularly in Latin America, with the current available techniques. Therefore, new diagnostic tools and predictors for poor outcome are imperative toward allowing the application of new therapeutic tools in those children at high risk of HUS development.

References

Ahmed A, Li J, Shiloach Y, Robbins JB, Szu SC (2006) Safety and immunogenicity of *Escherichia coli* O157 O-specific polysaccharide conjugate vaccine in 2–5 year-old children. J Infect Dis 193:515–521

Ahn CK, Klein E, Tarr PI (2008) Isolation of patients acutely infected with *Escherichia coli* O157:H7: low-tech, highly effective prevention of hemolytic uremic syndrome. Clin Infect Dis 46:1197–1199

Akiyoshi DE, Sheoran AS, Rich CM, Richard L, Chapman-Bonofiglio S, Tzipori S (2010) Evaluation of Fab and $F(ab')_2$ fragments and isotype variants of a recombinant human monoclonal antibody against Shiga toxin 2. Infect Immun 78:1376–1382

Allen K, Rogan D, Finlay B, Potter A, Asper D (2011) Vaccination with type III secreted proteins leads to decreased shedding in calves after experimental infection with *Escherichia coli* O157. Can J Vet Res 75:98–105

Behrens RH, Cramer JP, Jelinek T, Shaw H, von Sonnenburg F, Wilbraham D, Weinke T, Bell DJ, Astuias E, Pauwells HL, Maxwell R, Paredes-Paredes M, Glenn GM, Dewasthaly S, Stablein DM, Jiang ZD, Dupont HL (2014) Efficacy and safety of a patch vaccine containing heat labile toxin from *Escherichia coli* against travelers' diarrhoea: a phase 3, randomized, double-blind, placebo-controlled field trial in travelers from Europe to Mexico and Guatemala. Lancet Infect Dis 14:197–204

Bentancor LV, Bilen M, Brando RJ, Ramos MV, Ferreira LC, Ghiringhelli PD, Palermo MS (2009) A DNA vaccine encoding the enterohemorrhagic *Escherichia coli* Shiga-like toxin 2 A2 and B subunits confers protective immunity to Shiga toxin challenge in the murine model. Clin Vaccine Immunol 16:712–718

Berry ED, Wells JE (2012) Soil solarization reduces *Escherichia coli* O157: H7 and total *Escherichia coli* on cattle feedlot pen surfaces. J Food Prot 75:7–13

Bielaszewska M, Idelevich EA, Zhang W, Bauwens A, Schaumburg F, Mellmann A, Peters G, Karch H (2012) Effects of antibiotics on Shiga toxin 2 production and bacteriophage induction by epidemic *Escherichia coli* O104:H4 strain. Antimicrob Agents Chemother 56:3277–3282

Bitzan M, Poole R, Mehran M, Sicard E, Brockus C, Thuning-Roberson C, Rivière M (2009) Safety and pharmacokinetics of chimeric anti-Shiga toxin 1 and anti-Shiga toxin 2 monoclonal antibodies in healthy volunteers. Antimicrob Agents Chemother 53:3081–3087

Boisen N, Scheutz F, Rasko DA et al (2012) Genomic characterization of enteroaggregative *Escherichia coli* from children in Mali. J Infect Dis 205(3):431–444

Bourgeois AL, Wierzba TF, Walker RI (2016) Status of vaccine research and development for enterotoxigenic *Escherichia coli*. Vaccine 34:2880–2886

Burlaka I, Liu XL, Rebetz J, Arvidsson I, Yang L, Brismar H, Karpman D, Aperia A (2013) Ouabain protects against Shiga toxin-triggered apoptosis by reversing the imbalance between Bax and Bcl-xL. J Am Soc Nephrol 24:1413–1423

Cai K, Gao X, Li T, Hou X, Wang Q, Liu H, Xiao L, Tu W, Liu Y, Shi J, Wang H (2010) Intragastric immunization of mice with enterohemorrhagic *Escherichia coli* O157:H7 bacterial ghosts reduces mortality and shedding and induces a Th2-type dominated mixed immune response. Can J Microbiol 56:389–398

Cai K, Gao X, Li T, Wang Q, Hou X, Tu W, Xiao L, Tian M, Liu Y, Wang H (2011) Enhanced immunogenicity of a novel Stx2Am-Stx1B fusion protein in a mice model of enterohemorrhagic *Escherichia coli* O157:H7 infection. Vaccine 29:946–952

Callaway TR, Carr MA, Edrington TS, Anderson RC, Nisbet DJ (2009) Diet, *Escherichia coli* O157:H7, and cattle: a review after 10 years. Curr Issues Mol Biol 11:67–79

Carey CM, Kostrzynska M, Ojha S, Thompson S (2008) The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic *Escherichia coli* O157:H7. J Microbiol Methods 73:125–132

Chen YP, Lee TY, Hong WS, Hsieh HH, Chen MJ (2013) Effects of *Lactobacillus kefiranofaciens* M1 isolated from kefir grains on enterohemorrhagic *Escherichia coli* infection using mouse and intestinal cell models. J Dairy Sci 96:7467–7477

Cheng LW, Henderson TD, Patfield S, Stanker LH, He X (2013) Mouse in vivo neutralization of *Escherichia coli* Shiga toxin 2 with monoclonal antibodies. Toxins 5:1845–1858

Choi KS, Kim SH, Kim ED, Lee SH, Han SJ, Yoon S, Chang KT, Seo KY (2014) Protection from hemolytic uremic syndrome by eye drop vaccination with modified enterohemorrhagic *E. coli* outer membrane vesicles. PLoS One 9:e100229

Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, Huda N, Khan MU, Kay BA, Khan MR (1988) Cross-protection by B subunitwhole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J Infect Dis 158:372–7

Crane JK, Byrd IW, Boedeker EC (2011) Virulence inhibition by zinc in Shiga-toxigenic *Escherichia coli*. Infect Immun 79:1696–1705

Darsley MJ, Chakraborty S, DeNearing B, Sack DA, Feller A, Buchwaldt C, Bourgeois AL, Walker RI, Harro CD (2012) The oral, live attenuated enterotoxigenic *Escherichia coli* vaccine ACE527 reduces the incidence and severity of diarrhea in a human challenge model of diarrheal disease. Clin Vaccine Immunol 19:1921–1931

Del Canto F, Valenzuela P, Cantero L, Bronstein J, Blanco JE, Blanco J, Prado V, Levine M, Nataro J, Sommerfelt H, Vidal R (2011) Distribution of classical and non classical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J Clin Microbiol 49:3198–3203

Dowling TC, Chavaillaz PA, Young DG, Melton-Celsa A, O'Brien A, Thuning-Roberson C, Edelman R, Tacket CO (2005) Phase 1 safety and pharmacokinetic study of chimeric murine-human monoclonal antibody c alpha Stx2 administered intravenously to healthy adult volunteers. Antimicrob Agents Chemother 49:1808–1812

Dupont HL, Jiang ZD, Belkind-Gerson J, Okhuysen PC, Ericsson CD, Ke S, Huang DB, Dupont MW, Adachi JA, De La Cabada FJ, Taylor DN, Jaini S, Martinez Sandoval F (2007) Treatment of travelers' diarrhea: randomized trial comparing rifaximin, rifaximin plus loperamide, and loperamide alone. Clin Gastroenterol Hepatol 5:451–456

Dupont HL, Petersen A, Zhao J, Mundt A, Jiang ZD, Miller S, Flores J, Shringarpure R, Moro L, Bagin RG, Ballard ED, Totoritis MC (2014) Targeting of rifamycin SV to the colon for treatment of travelers' diarrhea: a randomized, double-blind, placebo-controlled phase 3 study. J Travel Med 21:369–376

El-Kamary SS, Cohen MB, Bourgeois AL, Van De Verg L, Bauers N, Reymann M, Pasetti MF, Chen WH (2013) Safety and immunogenicity of a single oral dose of recombinant double mutant heat-labile toxin derived from enterotoxigenic *Escherichia coli*. Clin Vaccine Immunol 20:1764–1770

Ellingsworth LR (2011) Transcutaneous immunization and the travelers' diarrhea vaccine system: a phase III pivotal efficacy study. In: 6th international conference on vaccines for enteric diseases, VED 2011, Cannes, France

Evans DG, Evans DJ Jr, Opekun AR, Graham DY (1988) Non-replicating oral whole cell vaccine protective against enterotoxigenic *Escherichia coli* (ETEC) diarrhea: stimulation of anti-CFA (CFA/I) and anti-enterotoxin (anti-LT) intestinal IgA and protection against challenge with ETEC belonging to heterologous serotypes. FEMS Microbiol Immunol 1:117–25

Fernandez-Brando RJ, Yamaguchi N, Tahoun A, McAteer SP, Gillespie T, Wang D, Argyle SA, Palermo MS, Gally DL (2016) Type III secretion-dependent sensitivity of *Escherichia coli* O157 to specific ketolides. Antimicrob Agents Chemother 60:459–470

Fleckenstein JM, Sheikh A, Qadri F (2014) Novel antigens for enterotoxigenic *Escherichia coli* vaccines. Expert Rev Vaccines 13:631–639

Flores J, Dupont HL, Jiang ZD, Belkind-Gerson J, Mohamed JA, Carlin LG, Padda RS, Paredes M, Martinez-Sandoval JF, Villa NA, Okhuysen PC (2008) Enterotoxigenic *Escherichia coli* heat-labile toxin seroconversion in US travelers to Mexico. J Travel Med 15:156–161

Flores J, Dupont HL, Jiang ZD, Okhuysen PC, Melendez-Romero JH, Gonzalez-Estrada A, Carrillo I, Paredes M (2011) A randomized, double-blind, pilot study of rifaximin 550 mg versus placebo in the prevention of travelers' diarrhea in Mexico during the dry season. J Travel Med 18:333–336

Flores-Figueroa J, Okhuysen PC, von Sonnenburg F, DuPont HL, Libman MD, Keystone JS, Hale DC, Burchard G, Han PV, Wilder-Smith A, Freedman DO (2011) Patterns of illness in travelers visiting Mexico and Central America: the GeoSentinel experience. Clin Infect Dis 53:523–531

Franz E, Veenman C, van Hoek AH, de RodaHusman A, Blaak H (2015) Pathogenic *Escherichia coli* producing extended-spectrum β-lactamases isolated from surface water and wastewater. Sci Rep 5:14372

Frech SA, Dupont HL, Bourgeois AL, McKenzie R, Belkind-Gerson J, Figueroa JF, Okhuysen PC, Guerrero NH, Martinez-Sandoval FG, Melendez-Romero JH, Jiang ZD, Asturias EJ, Halpern J, Torres OR, Hoffman AS, Villar CP, Kassem RN, Flyer DC, Andersen BH, Kazempour K, Breisch SA, Glenn GM (2008) Use of a patch containing heat labile toxin from *Escherichia coli* against travelers' diarrhoea: a phase II, randomized, double-blind, placebo-controlled field trial. Lancet 371:2019–2025

Friedrich AW, Bielaszewska M, Zhang WL, Pulz M, Kuczius T, Ammon A, Karch H (2002) *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis 185:74–84

Fujii J, Naito M, Yutsudo T, Matsumoto S, Heatherly DP, Yamada T, Kobayashi H, Yoshida S, Obrig T (2012) Protection by a recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin vaccine expressing Shiga toxin 2 B subunit against Shiga toxin- producing *Escherichia coli* in mice. Clin Vaccine Immunol 19:1932–1937

Gaastra W, Sommerfelt H, van Dijk L, Kusters JG, Svennerholm AM, Grewal HM (2002) Antigenic variation within the subunit protein of members of the colonization factor antigen I group of fimbrial proteins in human enterotoxigenic *Escherichia coli*. Int J Med Microbiol 292:43–50

Gao X, Cai K, Li T, Wang Q, Hou X, Tian R, Liu H, Tu W, Xiao L, Fang L, Luo S, Liu Y, Wang H (2011) Novel fusion protein protects against adherence and toxicity of enterohemorrhagic *Escherichia coli* O157:H7 in mice. Vaccine 29:6656–6663

Garcia-Angulo VA, Kalita A, Torres AG (2013) Advances in the development of enterohemorrhagic *Escherichia coli* vaccines using murine models of infection. Vaccine 31:3229–3235

Glenn GM, Villar CP, Flyer DC, Bourgeois AL, McKenzie R, Lavker RM, Frech SA (2007) Safety and immunogenicity of an enterotoxigenic *Escherichia coli* vaccine patch containing heat-labile toxin: use of skin pretreatment to disrupt the stratum corneum. Infect Immun 75:2163–2170

Gohar A, Abdeltawab NF, Fahmy A, Amin MA (2016) Development of safe, effective and immunogenic vaccine candidate for diarrheagenic *Escherichia coli* main pathotypes in a mouse model. BMC Res Notes 9:1–18

Güereña-Burgueño F, Hall ER, Taylor DN, Cassels FJ, Scott DA, Wolf MK, Roberts ZJ, Nesterova GV, Alving CR, Glenn GM (2002) Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. Infect Immun 70:1874–1880

Gu J, Liu Y, Yu S, Wang H, Wang Q, Yi Y, Zhu F, Yu XJ, Zou Q, Mao X (2009) Enterohemorrhagic *Escherichia coli* trivalent recombinant vaccine containing EspA, intimin and Stx2 induces strong humoral immune response and confers protection in mice. Microbes Infect 11:835–841

Gu J, Ning Y, Wang H, Xiao D, Tang B, Luo P, Cheng Y, Jiang M, Li N, Zou Q, Mao X (2011) Vaccination of attenuated EIS-producing *Salmonella* induces protective immunity against enterohemorrhagic *Escherichia coli* in mice. Vaccine 29:7395–7403

Gupta P, Singh MK, Singh Y, Gautam V, Kumar S, Kumar O, Dhaked RK (2011) Recombinant Shiga toxin B subunit elicits protection against Shiga toxin via mixed Th type immune response in mice. Vaccine 29:8094–8100

Harris JA, Roy K, Woo-Rasberry V, Hamilton DJ, Kansal R, Qadri F, Fleckenstein JM (2011) Directed evaluation of enterotoxigenic *Escherichia coli* autotransporter proteins as putative vaccine candidates. PLoS Negl Trop Dis 5:1–11

Harro C, Sack D, Bourgeois AL, Walker R, Denearing B, Feller A, Chakraborty S, Buchwaldt C, Darsley MJ (2011) A combination vaccine consisting of three live attenuated enterotoxigenic *Escherichia coli* strains expressing a range of colonization factors and heat-labile toxin subunit B is well tolerated and immunogenic in a placebo-controlled double-blind phase I trial in healthy adults. Clin Vaccine Immunol 18:2118–2127

Harro C, Gutierrez R, Talaat K, Porter C, Riddle M, Maciel M, et al (2015) Protective efficacy of an enterotoxigenic *E. coli* fimbrial tip adhesin vaccine given with LTR192G by intradermal vaccination against experimental challenge with CFA/I-ETEC in adult volunteers. Presented at the 3rd skin vaccination summit

Hauswaldt S, Nitschke M, Sayk F, Solbach W, Knobloch JK (2013) Lessons learned from outbreaks of Shiga toxin producing *Escherichia coli*. Curr Infect Dis Rep 15:4–9

Holmgren J, Bourgeois L, Carlin N, Clements J, Gustafsson B, Lundgren A, Nygren E, Tobias J, Walker RI, Svennerholm AM (2013) Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant. Vaccine 31:2457–2464

Hostetter SJ, Helgerson AF, Paton JC, Paton AW, Cornick NA (2014) Therapeutic use of a receptor mimic probiotic reduces intestinal Shiga toxin levels in a piglet model of hemolytic uremic syndrome. BMC Res Notes 7:331. doi:10.1186/17560500-7-331

Laird R, Gutierrez R, Savarino SJ, Riddle M, Maciel M (2014) Phenotypic and functional evaluation of T cell responses after intradermal immunization with LTR192G, an attenuated form of the enterotoxigenic *E. coli* (ETEC) labile toxin (LT), as an adjuvant for an ETEC vaccine. Presented at the American association of immunologist annual meeting

Larzábal M, Zotta E, Ibarra C, Rabinovitz BC, Vilte DA, Mercado EC, Cataldi A (2013) Effect of coiled-coil peptides on the function of the type III secretion system-dependent activity of enterohemorragic *Escherichia coli* O157:H7 and *Citrobacter rodentium*. Int J Med Microbiol 303:9–15

Li X, Wu P, Cheng S, Lv X (2012) Synthesis and assessment of globotriose-chitosan conjugate, a novel inhibitor of Shiga toxins produced by *Escherichia coli*. J Med Chem 55:2702–2710

López EL, Contrini MM, Falaschi A, Inchaurregui E, Galindez R, Ezcurra G, Villena R, Teplitz E, Acuña M, Gonzalez Ayala S, Vergara R, Twele L, Figueroa G, Mellman A, DiLópez EL, Contrini MM, Glatstein E, González Ayala S, Santoro R, Allende D, Ezcurra G, Teplitz E, Koyama T, Matsumoto Y, Sato H, Sakai K, Hoshide S, Komoriya K, Morita T, Harning R, Brookman S (2010) Safety and pharmacokinetics of urtoxazumab, a humanized monoclonal antibody, against Shiga-like toxin 2 in healthy adults and in pediatric patients infected with Shiga-like toxin-producing *Escherichia coli*. Antimicrob Agents Chemother 54:239–243

López EL, Contrini MM, Falaschi A, Inchaurregui E, Galindez R, Ezcurra G, Villena R, Teplitz E, Acuña M, Gonzalez Ayala S, Vergara R, Twele L, Figueroa G, Mellman A, Distabile L, Reymond D (2015) A phase II study of chimeric monoclonal antibodies to Shiga toxins 1 and 2 administered concomitantly to children with Shiga toxin producing *Escherichia coli* infection and bloody diarrhea (SHIGATEC trial). In: Abstracts of the 9th international symposium

on Shiga toxin (verocytotoxin) producing *Escherichia coli* infections, Boston, MA, 13–16 September 2015

Lu X, Skurnik D, Pozzi C, Roux D, Cywes-Bentley C, Ritchie JM, Munera D, Gening ML, Tsvetkov YE, Nifantiev NE, Waldor MK, Pier GB (2014) A poly-N-acetylglucosamine–Shiga toxin broad-spectrum conjugate vaccine for Shiga toxin-producing *Escherichia coli*. mBio 5:e00974-14

Lundgren A, Leach S, Tobias J, Carlin N, Gustafsson B, Jertborn M, Bourgeois L, Walker RI, Holmgren J, Svennerholm AM (2013) Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I over-expressing bacteria and recombinantly produced LTB/CTB hybrid protein. Vaccine 31:1163–1170

Luo Q, Kumar P, Vickers TJ, Sheikh A, Lewis WG, Rasko DA, Sistrunk J, Fleckenstein JM (2014) Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect Immun 82:509–521

McKenzie R, Bourgeois AL, Frech SA, Flyer DC, Bloom A, Kazempour K, Glenn GM (2007) Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. Vaccine 25:3684–3691

McNeilly TN, Mitchell MC, Corbishley A, Nath M, Simmonds H, McAteer SP, Mahajan A, Low JC, Smith DG, Huntley JF, Gally DL (2015) Optimizing the protection of cattle against *Escherichia coli* O157:H7 colonization through immunization with different combinations of H7 flagellin, Tir, intimin-531 or EspA. PLoS One 10(5):e0128391

Mejias MP, Ghersi G, Craig PO, Panek CA, Bentancor LV, Baschkier A, Goldbaum FA, Zylberman V, Palermo MS (2013) Immunization with a chimera consisting of the B subunit of Shiga toxin type 2 and *Brucella* lumazine synthase confers total protection against Shiga toxins in mice. J Immunol 191:2403–2411

Mejias MP, Hiriart Y, Lauché C, Fernandez-Brando RJ, Pardo R, Bruballa A, Ramos MV, Goldbaum FA, Palermo MS, Zylberman V (2016) Development of camelid single chain antibodies against Shiga toxin type 2 (Stx2) with therapeutic potential against Hemolytic Uremic Syndrome (HUS). Sci Rep 6:24913

Melton-Celsa AR, O'Brien AD (2013) New therapeutic developments against Shiga toxin producing *Escherichia coli*. Microbiol Spectr 2(5)

Moriel DG, Bertoldi I, Spagnuolo A, Marchi S, Rosini R, Nesta B, Pastorello I, Corea VA, Torricelli G, Cartocci E, Savino S, Scarselli M, Dobrindt U, Hacker J, Tettelin H, Tallon LJ, Sullivan S, Wieler LH, Ewers C, Pickard D, Dougan G, Fontana MR, Rappuoli R, Pizza M, Serino L (2010) Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*. Proc Natl Acad Sci U S A 107:9072–9077

Mukhopadhyay S, Linstedt AD (2012) Manganese blocks intracellular trafficking of Shiga toxin and protects against Shiga toxicosis. Science 335:332–335

Mulvey GL, Marcato P, Kitov PI, Sadowska J, Bundle DR, Armstrong GD (2003) Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. J Infect Dis 187:640–649

Nesta B, Spraggon G, Alteri C, Moriel DG, Rosini R, Veggi D, Smith S, Bertoldi I, Pastorello I, Ferlenghi I, Fontana MR, Frankel G, Mobley HL, Rappuoli R, Pizza M, Serino L, Soriani M (2012) FdeC, a novel broadly conserved *Escherichia coli* adhesin eliciting protection against urinary tract infections. MBio 3:e00010–1

Nishikawa K, Matsuoka K, Watanabe M, Igai K, Hino K, Hatano K, Yamada A, Abe N, Terunuma D, Kuzuhara H, Natori Y (2005) Identification of the optimal structure required for a Shiga toxin neutralizer with oriented carbohydrates to function in the circulation. J Infect Dis 191:2097–2105

Norton EB, Lawson LB, Freytag LC, Clements JD (2011) Characterization of a mutant *Escherichia coli* heat-labile toxin, LT (R192G/L211A), as a safe and effective oral adjuvant. Clin Vaccine Immunol 18:546–551

Otto W, Najnigier B, Stelmasiak T, Robins-Browne RM (2011) Randomized control trials using a tablet formulation of hyperimmune bovine colostrum to prevent diarrhea caused by enterotoxigenic *Escherichia coli* in volunteers. Scand J Gastroenterol 46:862–868

Ouyang-Latimer J, Jafri S, VanTassel A, Jiang ZD, Gurleen K, Rodriguez S, Nandy RK, Ramamurthy T, Chatterjee S, McKenzie R, Steffen R, DuPont HL (2011) In vitro antimicrobial susceptibility of bacterial enteropathogens isolated from international travelers to Mexico, Guatemala, and India from 2006 to 2008. Antimicrob Agents Chemother 55:874–878

Palermo MS, Exeni R, Fernandez GC (2009) Interventions in hemolytic uremic syndrome (HUS), the major Shiga toxin-related complication in *Escherichia coli* O157:H7 infections. Expert Rev Anti Infect Ther 7:697–707

Paredes-Paredes M, Okhuysen PC, Flores J, Mohamed JA, Padda RS, Gonzalez-Estrada A, Haley CA, Carlin LG, Nair P, DuPont HL (2011a) Seasonality of diarrheagenic *Escherichia coli* pathotypes in the US students acquiring diarrhea in Mexico. J Travel Med 18:121–125

Paredes-Paredes M, Flores-Figueroa J, Dupont HL (2011b) Advances in the treatment of travelers' diarrhea. Curr Gastroenterol Rep 13:402–407

Paton AW, Chen AY, Wang H, McAllister LJ, Höggerl F, Mayr UB, Shewell LK, Jennings MP, Morona R, Lubitz W, Paton JC (2015) Protection against Shiga-toxigenic *Escherichia coli* by non-genetically modified organism receptor mimic bacterial ghosts. Infect Immun 83:3526–3533

Qadri F, Wenneras C, Ahmed F, Asaduzzaman M, Saha D, Albert MJ, Sack RB, Svennerholm A (2000) Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children. Vaccine 18:2704–2712

Rasko DA, Moreira CG, de Li R, Reading NC, Ritchie JM, Waldor MK, Williams N, Taussig R, Wei S, Roth M, Hughes DT, Huntley JF, Fina MW, Falck JR, Sperandio V (2008) Targeting QseC signaling and virulence for antibiotic development. Science 321:1078–1080

Ren W, Yu R, Liu G, Li N, Peng Y, Wu M, Yin Y, Li Y, Fatufe AA, Li T (2013) DNA vaccine encoding the major virulence factors of Shiga toxin type 2e(Stx2e)-expressing *Escherichia coli* induces protection in mice. Vaccine 31:367–372

Riddle MS, Savarino SJ (2014) Moving beyond a heat-labile enterotoxin based vaccine against enterotoxigenic *Escherichia coli*. Lancet Infect Dis 14:174–175

Ritchie JM, Greenwich JL, Davis BM, Bronson RT, Gebhart D, Williams SR, Martin D, Scholl D, Waldor MK (2011) An *Escherichia coli* O157-specific engineered pyocin prevents and ameliorates infection by *E. coli* O157:H7 in an animal model of diarrheal disease. Antimicrob Agents Chemother 55:5469–5474

Rivas M, Chinen I, Miliwebsky E, Masana M (2014) Risk factors for Shiga toxin-producing *Escherichia coli*-associated human diseases. Microbiol Spectr 2(5). doi:10.1128/microbiolspec.EHEC-0002-2013

Rojas RL, Gomes PA, Bentancor LV, Sbrogio-Almeida ME, Costa SO, Massis LM, Ferreira RC, Palermo MS, Ferreira LC (2010) *Salmonella enteric* serovar Typhimurium vaccine strains expressing a nontoxic Shiga-like toxin 2 derivative induce partial protective immunity to the toxin expressed by enterohemorrhagic *Escherichia coli*. Clin Vaccine Immunol 17:529–536

Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, Dell'Orto V, Coddens A, Cox E, Fogher C (2013) Expression of verocytotoxic *Escherichia coli* antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model. J Vet Sci 14:263–270

Roy K, Hamilton D, Ostmann MM, Fleckenstein JM (2009a) Vaccination with EtpA glycoprotein or flagellin protects against colonization with enterotoxigenic *Escherichia coli* in a murine model. Vaccine 27:4601–4608

Roy K, Hilliard GM, Hamilton DJ, Luo J, Ostmann MM, Fleckenstein JM (2009b) Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. Nature 457(7229):594–598

Roy K, Kansal R, Bartels SR, Hamilton DJ, Shaaban S, Fleckenstein JM (2011) Adhesin degradation accelerates delivery of heat-labile toxin by enterotoxigenic *Escherichia coli*. J Biol Chem 286(34):29771–29779

Roy K, Hamilton DJ, Fleckenstein JM (2012) Cooperative role of antibodies against heat-labile toxin and the EtpA adhesin in preventing toxin delivery and intestinal colonization by enterotoxigenic *Escherichia coli*. Clin Vaccine Immunol 19:1603–1608

Ruan X, Knudsen DE, Wollenberg KM, Sack DA, Zhang W (2014a) Multiepitope fusion antigen induces broadly protective antibodies that prevent adherence of *Escherichia coli* strains expressing colonization factor antigen I (CFA/I), CFA/II, and CFA/IV. Clin Vaccine Immunol 21:243–249

Ruan X, Robertson DC, Nataro JP, Clements JD, Zhang W, STa Toxoid Vaccine Consortium Group (2014b) Characterization of heat-stable (STa) toxoids of enterotoxigenic *Escherichia coli* fused to double mutant heat labile toxin peptide in inducing neutralizing anti-STa antibodies. Infect Immun 82:1823–1832

Ruan X, Sack DA, Zhang W (2015) Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) retain broad anti-CFA and antitoxin antigenicity. PLoS One 10, e0121623

Rund SA, Rohde H, Sonnenborn U, Oelschlaeger TA (2013) Antagonistic effects of probiotic *Escherichia coli* Nissle 1917 on EHEC strains of serotype O104:H4 and O157:H7. Int J Med Microbiol 303:1–8

Sack DA, Shimko J, Torres O, Bourgeois AL, Francia DS, Gustafsson B, Karnell A, Nyquist I, Svennerholm AM (2007) Randomized, double-blind, safety and efficacy of a killed oral vaccine for enterotoxigenic *E. coli* diarrhoea of travelers to Guatemala and Mexico. Vaccine 25:4392–4400

Sahl JW, Steinsland H, Redman JC (2011) A comparative genomic analysis of diverse clonal types of enterotoxigenic *Escherichia coli* reveals pathovar-specific conservation. Infect Immun 79:950–960

Sato T, Matsui T, Takita E, Kadoyama Y, Makino S, Kato K, Sawada K, Hamabata T (2013) Evaluation of recombinant forms of the Shiga toxin variant Stx2eB subunit and non-toxic mutant Stx2e as vaccine candidates against porcine edema disease. J Vet Med Sci 75:1309–1315

Savarino SJ, Hall ER, Bassily S, Wierzba TF, Youssef FG, Peruski LF Jr, Abu-Elyazeed R, Rao M, Francis WM, El Mohamady H, Safwat M, Naficy AB, Svennerholm AM, Jertborn M, Lee YJ, Clemens JD (2002) Introductory evaluation of an oral, killed whole cell enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Egyptian infants. Pediatr Infect Dis J 21:322–330

Sheng H, Knecht HJ, Kudva IT, Hovde CJ (2006) Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. Appl Environ Microbiol 72:5359–5366

Sheoran AS, Dmitriev IP, Kashentseva EA, Cohen O, Mukherjee J, Debatis M, Shearer J, Tremblay JM, Beamer G, Curiel DT, Shoemaker CB, Tzipori S (2015) Adenovirus vector expressing Stx1/Stx2-neutralizing agent protects piglets infected with *Escherichia coli* O157:H7 against fatal systemic intoxication. Infect Immun 83:286–291

Silberstein C, Lucero MS, Zotta E, Copeland DP, Lingyun L, Repetto HA, Ibarra C (2011) A glucosylceramide synthase inhibitor protects rats against the cytotoxic effects of Shiga toxin 2. Pediatr Res 69:390–394

Sillankorva SM, Oliveira H, Azeredo J (2012) Bacteriophages and their role in food safety. Int J Microbiol 2012:863945

Smith MJ, Teel LD, Carvalho HM, Melton-Celsa AR, O'Brien AD (2006) Development of a hybrid Shiga holotoxoid vaccine to elicit heterologous protection against Shiga toxins types 1 and 2. Vaccine 24:4122–4129

Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Clowser SL (2008) A two-dose regimen of a vaccine against *Escherichia coli* O157:H7 type III secreted proteins reduced environmental transmission of the agent in a large-scale commercial beef feedlot clinical trial. Foodborne Pathog Dis 5:589–598

Snedeker KG, Campbell M, Sargeant JM (2012) A systematic review of vaccinations to reduce the shedding of *Escherichia coli* O157 in the faeces of domestic ruminants. Zoonoses Public Health 59:126–138

Soh HS, Chung HY, Lee HH, Ajjappala H, Jang K, Park JH, Sim JS, Lee GY, Lee HJ, Han YH, Lim JW, Choi I, Chung IS, Hahn BS (2015) Expression and functional validation of heat-labile enterotoxin B (LTB) and cholera toxin B (CTB) subunits in transgenic rice (Oryza sativa). Springerplus 4:148

Stanford K, Bach S, Baah J, McAllister T (2014) A mixture of *Lactobacillus casei*, *Lactobacillus lactis*, and *Paenibacillus polymyxa* reduces *Escherichia coli* O157:H7 in finishing feedlot cattle. J Food Prot 77:738–744

Steel D, Riddle M, Van De Verg L, Bourgeois AL (2012) Vaccine for enteric diseases: a meeting summary. Exp Rev Vaccine 11:407–9

Steffen R, Cramer JP, Burchard G, Jelinek T, Schwarz U, Ramdas P, Chatterjee C, Jiang ZD, DuPont HL, Dewasthaly S, Westrischnig K, Behrens RH (2013) Efficacy of a travelers' diarrhea vaccine system in travelers to India. J Travel Med 20:374–379

Steffen R, Hill DR, DuPont HL (2015) Traveler's diarrhea: a clinical review. JAMA 313:71–80

Stone SM, Thorpe CM, Ahluwalia A, Rogers AB, Obata F, Vozenilek A, Kolling GL, Kane AV, Magun BE, Jandhyala DM (2012) Shiga toxin 2-induced intestinal pathology in infant rabbits is A-subunit dependent and responsive to the tyrosine kinase and potential ZAK inhibitor imatinib. Front Cell Infect Microbiol 2:135

Svennerholm AM, Tobias J (2008) Vaccines against enterotoxigenic *Escherichia coli*. Exp Rev Vaccine 7:795–804

Szu SC, Ahmed A (2014) Clinical studies of *Escherichia coli* O157:H7 conjugate vaccines in adults and young children. Microbiol Spectr 2(6). doi:10.1128/microbiolspec.EHEC-0016-2013

Takeyama N, Kiyono H, Yuki Y (2015) Plant-based vaccines for animals and humans: recent advances in technology and clinical trials. Ther Adv Vaccine 3:139–54

Taylor CM, Bitzan M, Reymond D (2011) SHIGATEC: a phase II study assessing monoclonal antibodies against Shiga toxin 1 and 2 in Shiga toxin-producing *E. coli*-infected children. Pediatr Nephrol 26:1595–1596

Thomas DE, Elliott EJ (2013) Interventions for preventing diarrhea-associated hemolytic uremic syndrome: systematic review. BMC Public Health 13:799

Thomson DU, Loneragan GH, Thornton AB, Lechtenberg KF, Emery DA, Burkhardt DT, Nagaraja TG (2009) Use of a siderophore receptor and porin proteins-based vaccine to control the burden of *Escherichia coli* O157:H7 in feedlot cattle. Foodborne Pathog Dis 6:871–877

Trachtman H, Cnaan A, Christen E, Gibbs K, Zhao S, Acheson DW, Weiss R, Kaskel FJ, Spitzer A, Hirschman GH, Investigators of the HUS-SYNSORB Pk Multicenter Clinical Trial (2003) Effect of an oral Shiga toxin-binding agent on diarrhea-associated hemolytic uremic syndrome in children: a randomized controlled trial. JAMA 290:1337–1344

Tremblay JM, Mukherjee J, Leysath CE, Debatis M, Ofori K, Baldwin K, Boucher C, Peters R, Beamer G, Sheoran A, Bedenice D, Tzipori S, Shoemaker CB (2013) A single VHH-based toxin-neutralizing agent and an effector antibody protect mice against challenge with Shiga toxins 1 and 2. Infect Immun 81:4592–4603

Tsuji T, Shimizu T, Sasaki K, Tsukamoto K, Arimitsu H, Ochi S, Shimizu T, Taniguchi K, Noda M, Neri P, Mori H (2008) A nasal vaccine comprising B-subunit derivative of Shiga toxin 2 for cross-protection against Shiga toxin types 1 and 2. Vaccine 26:2092–2099

Turner AK, Terry TD, Sack DA, Londono-Arcila P, Darsley MJ (2001) Construction and characterization of genetically defined *aro omp* mutants of enterotoxigenic *Escherichia coli* and preliminary studies of safety and immunogenicity in humans. Infect Immun 69:4969–4979

Tzipori S, Sheoran A, Akiyoshi D, Donohue-Rolfe A, Trachtman H (2004) Antibody therapy in the management of Shiga toxin-induced hemolytic uremic syndrome. Clin Microbiol Rev 17:926–941

Vilte DA, Larzabal M, Garbaccio S, Gammella M, Rabinovitz BC, Elizondo AM, Cantet RJ, Delgado F, Meikle V, Cataldi A, Mercado EC (2011) Reduced faecal shedding of *Escherichia coli* O157:H7 in cattle following systemic vaccination with gamma-intimin C280 and EspB proteins. Vaccine 29:3962–3968

Walker RI (2015) An assessment of enterotoxigenic *Escherichia coli* and *Shigella* vaccine candidates for infants and children. Vaccine 33:954–965

Watanabe-Takahashi M, Sato T, Dohi T, Noguchi N, Kano F, Murata M, Hamabata T, Natori Y, Nishikawa K (2010) An orally applicable Shiga toxin neutralizer functions in the intestine to inhibit the intracellular transport of the toxin. Infect Immun 78:177–183

WHO (2006) Future directions for research on enterotoxigenic *Escherichia coli* vaccines in developing countries. WHO Wkly Epidemiol Rec 81:97–104

Wileman BW, Thomson DU, Olson KC, Jaeger JR, Pacheco LA, Bolte J, Burkhardt DT, Emery DA, Straub D (2011) *Escherichia coli* O157:H7 shedding in vaccinated beef calves born to cows vaccinated prepartum with *Escherichia coli* O157:H7 SRP vaccine. J Food Prot 74:1599–1604

Wong CS, Mooney JC, Brandt JR, Staples AO, Jelacic S, Boster DR, Watkins SL, Tarr PI (2012) Risk factors for the hemolytic uremic syndrome in children infected with *Escherichia coli* O157:H7: a multivariable analysis. Clin Infect Dis 55:33–41

Yu J, Cassels F, Scharton-Kersten T, Hammond SA, Hartman A, Angov E, Corthesy B, Alving C, Glenn GM (2002) Transcutaneous immunization using colonization factor and heat-labile enterotoxin induces correlates of protective immunity for enterotoxigenic *Escherichia coli*. Infect Immun 70:1056–1068

Zhang W, Sack DA (2015) Current progress in developing subunit vaccines against enterotoxigenic *Escherichia coli*-associated Diarrhea. Clin Vaccine Immunol 22:983–991

Zhang W, Zhang C, Francis DH, Fang Y, Knudsen D, Nataro JP, Rotertson DC (2010) Genetic fusions of heat-labile (LT) and heat-stable (ST) toxoids of porcine enterotoxigenic *Escherichia coli* elicit neutralizing anti-LT and anti-STa antibodies. Infect Immun 78:316–325

Zhang XH, He KW, Zhao PD, Ye Q, Luan XT, Yu ZY, Wen LB, Ni YX, Li B, Wang XM, Guo RL, Zhou JM, Mao AH (2012) Intranasal immunization with Stx2B-Tir-Stx1B-Zot protein leads to decreased shedding in goats after challenge with *Escherichia coli* O157:H7. Vet Rec 170:178

Zhang C, Knudsen DE, Liu M, Robertson DC, Zhang W, STa Toxoid Vaccine Consortium Group (2013) Toxicity and immunogenicity of enterotoxigenic *Escherichia coli* heat-labile and heat-stable toxoid fusion 3xSTa (A14Q)-LT (S63K/R192G/L211A) in a murine model. PLoS One 8(10):e77386

Chapter 12
Homeostasis vs. Dysbiosis: Role of Commensal *Escherichia coli* in Disease

Claudia F. Martinez de la Peña, Glen D. Armstrong, Margarita M.P. Arenas-Hernández, and Roberto J. Cieza

Summary Since the beginning, life on Earth has been influenced by the association between organisms and this continuous co-evolutionary process, led in life as we know it today. In this process, humans and microorganisms developed an intimate relationship known as the human microbiome. Any disturbance in our resident microbial community such as a shift away from homeostasis likely expresses itself in a pathological state. In this context, the term dysbiosis is used to describe a state of imbalance between the microbial population and the host species. *E. coli*, as part of our normal microbiome play an important role in homeostasis and it also has been linked to dysbiotic process. In the present chapter, we describe the role of different *E. coli* strains that have been linked to homeostasis and dysbiosis. Adherent Invasive *E. coli* (AIEC), lacking the known virulence factors of other pathogenic strains, is involved in inflammatory bowel disease (IBD). Enterohemorrhagic *E. coli,* together with the by-products of other members of the microbiome, colonize the human colon and *E. coli* Nissle is involved in maintaining homeostasis between host and its associated microbiome. The most recent studies in all Americas are also included in the present work.

C.F. Martinez de la Peña (✉) • M.M.P. Arenas-Hernández
Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias,
Benemérita Universidad Autónoma de Puebla, Puebla, México
e-mail: claudia.martinez@correo.buap.mx; maguie10@gmail.com

G.D. Armstrong
Department of Microbiology, Immunology and Infectious Diseases,
University of Calgary, Calgary, AB, Canada
e-mail: glen.armstrong@ucalgary.ca

R.J. Cieza
Department of Microbiology, Uniformed Services
University of the Health Sciences (USUHS), Bethesda, MD, USA
e-mail: robertocieza@gmail.com

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_12

1 General Concepts Related to Homeostasis and Dysbiosis in the Mammalian Gastrointestinal Tract

In a 2001 publication in The Scientist, Joshua Lederberg defined the microbiome as "*The totality of microorganisms and their collective genetic material present in or on the human body or in another environment*." (Lederberg and Mccray 2001). Since then, and with the advent of modern rapid gene sequencing and bioinformatics technologies, it is becoming clearer that the microbiome has considerable influence over the health and well-being of its host species. To fully understand the microbiome and its role in promoting sickness and health, it may be useful to first consider the evolutionary influences that have contributed to the current association between all animals and their compliment of unicellular microorganisms known collectively as their microbiome.

By current estimates, Earth is approximately 4.6 billion years old and, sometime between 3.9 and 2.5 billion years ago, it is speculated that complex organic molecules became organized and the first primitive unicellular life-forms appeared. During subsequent thousands of millennia, environmental conditions likely favored the establishment of communities of single microorganisms based on their mutualistic needs to access vital nutrients as well as protect themselves from hostile external influences. Early on, these ancestral poly-microbial communities would likely have assumed the structure and function of what are recognized today as biofilms. This process may have eventually led to the appearance of the first complex multi-cellular species, probably about 800 million years before present. During this time, successful unicellular organisms such as bacteria, fungi, and viruses continued exploiting environmental niches that sustained their growth and survival and, in many cases, these niches probably included communities in which unicellular individuals were becoming more structurally and functionally specialized. By this reasoning, it may be safely assumed that the evolution of complex multi-cellular life-forms occurred simultaneously and in harmony with all the unicellular microorganisms sharing their environment.

The continuing evolution of complex life-forms presented unicellular microorganisms with a growing menu of opportunities for enhancing their survival. In this regard, mutualism likely continued to play a major role in positively influencing the developing relationships. From the beginning; however, predator versus prey associations also likely co-evolved with those of the mutualistic variety again, mostly driven by competition for nutrient supplies and ideal environmental niches. Predator versus prey relationships probably began with the appearance of more advanced unicellular organisms such as amebae and paramecia that were equipped to forage on less advanced bacteria and fungi. This process was likely adopted as a successful survival strategy by complex multicellular species, including those that had established a mutualistic relationship with unicellular microorganisms, which now constituted their microbiomes.

In all these complex relationships of the mutualistic or adversarial variety, the overriding principal of homeostasis, or balanced coexistence, between individual

consenting members of the mutualistic community was essential to the survival of the whole being. Accordingly, the well-being of the complex host life-form was just as dependent on that of its microbiome as was the microbiome on its consenting host. The complex relationship that worked to ensure the survival of the community would be put in serious jeopardy, if one or several members suddenly adopted an adversarial position or were presented with an exploitive opportunity that would upset homeostasis. This need imposed an extra element to the successful evolution of mutualistic relationships in that, by necessity, each of the partners needed to always be vigilant of the intentions of the other members of the community and be able to avoid or counter any deviant behaviors that might jeopardize homeostasis.

Since the beginning, the evolution of all life on Earth has been influenced by close associations involving simple and more complex organisms and the checks and balances required to maintain harmony in mutualistic communities may have eventually produced a rudimentary type of immunity system to the benefit of all participants. Through the ages, this continuing co-evolutionary process culminated in the present, whereby all of Earth's creatures coexist in association with a microbiome composed of tens of billions of individual unicellular organisms. Because of the evolutionary process, these organisms are as much a part of us, as our other vital organs and systems, and we are as dependent on them as they are on us for our mutual well-being. As with any vital organ or system, a disturbance in our resident microbial community such as a shift away from homeostasis likely expresses itself in a pathological state.

But for a few exceptions, the development of the mammalian fetus occurs in the sterile environment of the uterus. As such, the co-evolutionary process that ensures individuals become colonized by a beneficial microbiome is delayed until birth. In practical terms, this means humans are not born with all of the fully functional vital organs and systems. This imposes an extra element in the developmental program since, by necessity, neonatal bodies need to acquire another vital component for survival after all other organ systems have become functional. In doing so, it is obligatory that the resident microbiome that we eventually acquire as youngsters and adults develops into a non-pathological state of homeostasis; a process which is dependent on all consenting partners transmitting, perceiving, and responding appropriately to important physiological and environmental cues, including a complex library of genetically encoded inflammatory and immunomodulating signaling molecules, immunocytes, hormones, and metabolic by-products, such as short chain fatty acids, and simple and complex carbohydrate sequences among others.

Such cues are produced by the cellular substrate (mucosal surface) in or on which the microbiome needs to become established, the host's response to colonization, and microbial by-products of growth and metabolism. It is an iterative two-way learning process which has evolved over many millennia in which both partners develop in harmony based on a close dialog involving all available cues. Under normal circumstances, the process leads to a long-term mutually beneficial homeostatic relationship. Should, however, one of the partners fail to emit the appropriate environmental cues or respond in an appropriate manner to the environmental cues, another homeostatic state will be established leading to a chronic pathological or

otherwise unhealthy condition. Accumulating evidence (Keely et al. 2015) suggests this may partly or entirely explain some chronic inflammatory bowel or other hyper-inflammatory or sensitivity syndromes.

The term dysbiosis is used to describe a state of imbalance between the microbial population and the host species. This can occur when the microbial population in or on the host is altered in its composition, species complexity, or total number of organisms. The resulting changes in the environmental cues expressed by the altered microbial population may be perceived as a threat or as a concerning change by the host, thereby establishing a pathogenic condition. For example, a limited course of antibiotics may lead to reversible dysbiosis resulting in transient pathogenesis followed by a return to the original state of homeostasis and health without further complications. Antibiotics, particularly if they are administered early in life, thereby intervening inappropriately in the delicate mutual host-microbiome dialog, may lead to an irreversible new state of homeostasis and chronic pathology. Similarly, alterations in host physiology caused by stress, dietary alterations, non-antimicrobial, or immunomodulating medications may also cause dysbiosis, resulting from changes in host-derived environmental cues. This may, in turn, lead to transient or chronic pathogenic conditions (Janka Babickova 2015). In principal, the factors governing the outcome of dysbiosis likely involve alterations in both host and microbial environmental cues, some of which are governed by host and microbial genetically encoded factors (de Lange and Barrett 2015; Mizoguchi et al. 2016).

Although many of the complex multifactorial influences governing the host–microbiome relationship are becoming clear, the influence of infectious microorganisms on the microbiome, particularly those capable of colonizing the gastrointestinal (GI) tract, is less well-established. *Escherichia coli* represent a good example since this group of organisms contains both benign as well as pathogenic members. Can *E. coli*, by means of genetic changes, adopt either a benign or adversarial position and, if so, what might be the pathological consequences of this behavioral shift on a host-microbiome relationship that originally developed as a healthy mutualistic relationship?

2 Recent Advances in Homeostasis vs. Dysbiosis in *E. coli* Research

Of considerable interest is the globally distributed enterovirulent group of *Escherichia coli*. These are especially singled out because the *E. coli* clan encompasses both normal, non-harmful components of the microbiome, in addition to closely related pathogenic strains. Therefore, a better understanding of how these organisms fit the complex host-microbiome evolutionary story could provide additional valuable insight into the aberrant development of their relationship, as it relates to chronic hypersensitivity and immunopathological syndromes of the GI and other mucosal systems. Although *E. coli* is part of the healthy human intestinal

microbiome, comprising 5 % of the total microbes that occupy the intestinal milieu (Consortium 2012), it is important to understand how this member, although not the most abundant, plays a role in gut dysbiosis.

One example of *E. coli* lacking the known virulence factors of other pathogenic strains but involved in inflammatory bowel disease (IBD) is Adherent Invasive *E. coli* (AIEC). IBD represents a collection of chronic GI disorders that are subdivided into two clinical manifestations, Crohn's disease (CD) and ulcerative colitis (UC). There is accumulating evidence that in IBD patients, there is an alteration of the microbiome (Frank et al. 2007). Moreover, there are significant differences between the microbiome composition of CD compared with UC patients (Sokol et al. 2006; Frank et al. 2007). In general, there is a marked reduction of the Gram-positive *Firmicutes* phylum and an increase of Gram-negative *Bacteroidetes* and facultative anaerobes such as *Enterobacteriaceae* (Frank et al. 2007). Although the cause or effect relationship of AIEC in CD is not known, it is clear that these bacteria play a role in developing pathogenesis of this condition. For a better comprehension of AIEC in IBD evolution, it is important to understand how AIEC is different from intestinal pathogenic *E. coli* and how the interaction with the host plays a role in this pathology.

AIEC is defined as an *E. coli* strain with the ability to: (a) adhere to differentiated Caco-2 and/or undifferentiated I-407 intestinal epithelial cells, (b) invade I-407 and HEp-2 cells, (c) induce host actin polymerization and microtubule recruitment, and (d) survive and replicate within J774-A1 macrophages (Darfeuille-Michaud et al. 2004). This *E. coli* strain was isolated from a chronic ileal CD lesion in an attempt to determine the reason for the elevated abundance of *E. coli* in this condition (Darfeuille-Michaud et al. 1998).

2.1 AIEC Adhesion

The ability of AIEC to adhere to intestinal epithelial cells (IECs) is mediated by the binding of the bacteria type 1 pili to the host glycoprotein carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) present in intestinal epithelial cells (Barnich 2007; Carvalho et al. 2009) (Fig. 12.1). It has been shown that the CEACAM6 receptor is abnormally expressed in CD patients and that the interaction of type 1 pili with this receptor is via FimH, the tip adhesin (Barnich 2007). The type 1 pilus is present in almost all *E. coli* strains and is known to bind mannose sugar receptor sequences found on host cell surfaces (Nilsson et al. 2006). A recent study demonstrated that FimH from 40 of 45 AIEC isolates carried hotspot mutations at positions G73, T158, and R166 and further analysis revealed that these mutations are from a recent evolutionarily origin and can become signatures of patho-adaptive mutations (Dreux et al. 2013). Among the FimH variants, there is one clade, S70N/N78S, that is associated with the B2 group and, interestingly, all AIEC strains belonging to that clade demonstrated a significantly higher ability to adhere to CEACAM6 expressed on undifferentiated T84 cells. Further, they are

more persistent in transgenic mice expressing human CEACAM6 than those strains expressing FimH from the consensus clade (found in non-AIEC strains) (Dreux et al. 2013). On the other hand, it was revealed that the FimH tip adhesin is also able to bind glycoprotein 2 (Gp2) present on the apical cell membrane of M cells within Peyer's Patches (PP) (Fig. 12.1), thereby favoring uptake and delivery of AIEC to mucosal immunity inductive sites in the GI tract (Hase et al. 2009).

Besides type 1 fimbriae, AIEC strains also express long polar fimbriae (Lpf). These fimbriae have been shown to be an important adherence factor for enterohemorrhagic *E. coli* and for *Salmonella* Typhimurium (Baumler et al. 1996; Torres et al. 2002, 2004; Lloyd et al. 2012). In AIEC, Lpf are involved in interactions with M cells at PP, but not with other intestinal epithelial cells (IEC) (Chassaing et al. 2011) (Fig. 12.1). Flagella, on the other hand, can affect adhesion in a direct or indirect manner. It has been shown that the *flhDC* gene products indirectly downregulate the expression of type 1 pili, consequently affecting bacterial adhesion (Barnich et al. 2003), and a *fliC* mutant of AIEC O83:H1 demonstrated diminished adhesion and invasion of Caco-2BBe and T-84 cells (Eaves-Pyles et al. 2008).

In 2015, Gibold et al. identified another factor involved in AIEC adherence, the Vat-AIEC autotransporter. The amino acid sequence analysis of this protein revealed that it is a serine protease, very similar to the Vat vacuolating toxin from avian pathogenic *E. coli* (APEC). This protein possesses mucolytic activity, helping AIEC to disrupt the mucus layer and favoring adherence to IEC. Furthermore, deletion of the Vat-AIEC encoding gene impairs AIEC adhesion in a murine intestinal loop

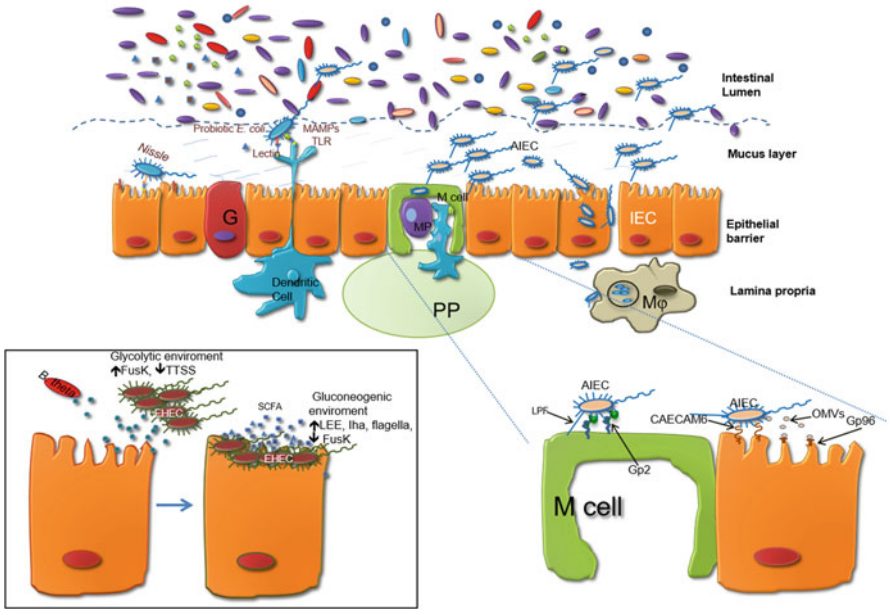

Fig. 12.1 Role of *E. coli* Nissle, AIEC, and EHEC in homeostasis and dysbiosis of the intestinal microbiome. *PP* Peyer's Patch, *IEC* intestinal epithelial cell, *Mϕ* macrophage, *TLR* toll like receptor, *G* globet cell

model (Gibold et al. 2015). The expression of Vat-AIEC is regulated by conditions such as a pH of 7.5, as well as bile salts and mucins found in the gut environment, influencing intestinal colonization by AIEC (Gibold et al. 2015).

2.2 AIEC Invasion

AIEC does not possess the canonical invasins that have been found in other enterobacteria; however, several studies have been conducted to explain its invasive phenotype. One of the mechanisms found to be involved in AIEC invasion is the use of outer membrane vesicles (OMV). AIEC produce OMV of 50–200 nm in diameter and are discharged from the bacteria during cell growth. It was shown that AIEC OMV are involved in bacterial invasion and that the OmpA and OmpC outer-membrane proteins formed part of these vesicles (Rolhion et al. 2005). The mechanism implicates the binding of OmpA protein to the host Gp96 protein (endoplasmic reticulum-localized stress response chaperone) (Fig. 12.1) present on the apical surface of ileal epithelial cells, allowing fusion of the OMV to the cell membrane (Rolhion et al. 2010). It has been suggested that binding of OMV to the cell releases unknown bacterial effectors involved in actin polymerization and microtubule recruitment. Moreover, it was found that CD patients overexpress the Gp96 protein in their ileal epithelial cells, predisposing them to AIEC colonization (Rolhion et al. 2010).

Another AIEC invasive mechanism involves the IbeA protein (invasion of the brain endothelium protein A). This invasin was first described in a newborn meningitis-causing *E. coli* (NMEC) isolate (Huang et al. 2001). Genomic analysis of the AIEC reference strains LF82 and NRG857c revealed that these strains also possess the *gimA* locus including *ibeA* (Cieza et al. 2015). Mutational analysis of *ibeA* gene revealed that its corresponding protein is involved in AIEC invasion of M and Caco-2 cells. However, invasion was not completely abolished in *ibeA* mutant strains, suggesting that other unknown invasins are also involved in this process. Furthermore, the mice employed in this work were conventional mice (no CEACAM6 transgenic mice), which resulted in the AIEC strains persisting more than nonpathogenic *E. coli*, suggesting that AIEC IbeA, in collaboration with other adhesins, is playing a role in the colonization phenotype (Cieza et al. 2015).

2.3 AIEC Intracellular Survival

It is well-known that AIEC can survive inside macrophages during in vitro and in vivo conditions (reviewed in (Martinez-Medina and Garcia-Gil 2014)). Moreover, they can also be found in the lamina propria and in the mesenteric lymph nodes (O'Brien et al. 2014). However, the bacterial mechanisms and the specific AIEC factors involved in intracellular survival are still unclear. It was found that the IbeA protein also plays a role in the early AIEC interaction with macrophages and

facilitates bacteria intracellular survival (Cieza et al. 2015). It has also been found that AIEC induces formation of a large vacuole inside the macrophage where it can replicate in the acidic environment (Bringer et al. 2006).

On the other hand, a recent study describes the role of GipA, a protein involved in *Salmonella* Typhimurium intracellular survival within PP, but also present in some *E. coli* strains associated with urinary and intestinal tract infections (Vazeille et al. 2016). GipA is expressed in some AIEC strains (LF82 and NRG857c) and is necessary for PP colonization and internalization, as demonstrated by mutational analysis of the *gipA* gene. This was observed in murine ileal loops containing, or not, one Peyer's patch and in vivo using human CEACAM6 expressed in transgenic mice. It has also been found that GipA positively regulates Lpf expression (Vazeille et al. 2016).

AIEC is considered a pathobiont because, despite the virulence factors it possesses, and although it has been found in IBD patients, it is also present in healthy subjects, forming part of their microbiome. AIEC in IBD is a good example of host–pathogen interaction, where not only the bacterial determinants play a role in inflammation, but also the environment and host susceptibility factors come into effect. For example, it has been found that there is a relation between the host NOD2 intracellular receptor and AIEC infection. NOD2 can sense intracellular pathogen-associated molecular patterns (PAMP), specifically the muramyl dipeptide of bacterial peptidoglycan, and is expressed by dendritic cells, macrophages/monocytes, and epithelial cells (Jensen et al. 2011). It has been shown that some polymorphisms in NOD2 are associated with an increased risk and severity of CD.

The single nucleotide polymorphism 13 (SNP13) NOD2 variant is associated with an increased risk of CD. This polymorphism produces a truncated NOD2 protein with altered function (Economou et al. 2004; Yazdanyar et al. 2009). Recently, it was determined that NOD2 interacts with autophagy-related 16-like-1 (ATG16L1) when the bacteria enter into the cell (Cooney et al. 2010; Travassos et al. 2010). This interaction relates autophagy with microbial processing inside the cells. Besides the polymorphisms in NOD2, it has been shown that CD predisposition includes polymorphisms in ATG16L1, immunity-related GTPase family M (IRGM), and interleukin 23. Furthermore, AIEC infection indirectly downregulates expression of ATG5 and ATG16L1, mediated by induction of high levels of 2 micro RNAs (MIR), MIR30C and MIR130A, by activation of NF-kB. Therefore, AIEC inhibits autophagy allowing increased survival of intracellular microorganisms and the subsequent increase in inflammatory responses (Nguyen et al. 2014).

2.4 AIEC Diversity

Two AIEC strains that have been used as reference isolates were obtained from the ileum of Crohn's Disease patients. The first one is LF82, which was the first AIEC described by Darfeuille-Michaud and collaborators (Darfeuille-Michaud 2002; Darfeuille-Michaud et al. 2004), and the second one by Torres and collaborators is NRG857c (Eaves-Pyles et al. 2008). Both strains have been fully sequenced by

different groups and this revealed some interesting features and similarities (Miquel et al. 2010; Nash et al. 2010). Both strains belong to the serotype O83:H1, are part of the B2 phylogenetic group, and they are phylogenetically related to extra-intestinal pathogenic *E. coli* (ExPEC), including some uropathogenic (UPEC) and neonatal meningitis *E. coli* (NMEC) as well as a close relationship with avian pathogenic strain APEC_01. Both of these strains possess large plasmids (108 kb and 150 kb, respectively) and the presence of several genomic islands. NRG857c possess 35 genomic islands and LF82 only nine large genomic islands (Miquel et al. 2010; Nash et al. 2010). Both AIEC also have the genomic ability to express two putative type VI secretion systems (T6SS).

Despite the similarities found in these two reference strains, many other AIEC strains have been isolated from IBD patients. Many research groups have been trying to identify genes that could be used for AIEC identification and, therefore, IBD diagnosis. AIEC strains isolated from IBD patients belong to a diverse group, and attempts to identify virulence factors have been challenging. A recent study using genomic analysis, compared the AIEC reference strain LF82 with the noninvasive *E. coli* (NIEC) strain HS and found that 747 coding DNA sequences (CDS) present in LF82 were absent in the NIEC strain. When comparing LF82 with another 11 NIEC strains, the number of CDS was much lower; finding only 166 CDS unique to LF82. Further, comparison of these 166 CDS among another 12 AIEC strains revealed that none of the 166 CDS were present in all of the 13 AIEC strains (Zhang et al. 2015). However, there were six genes with higher prevalence among LF82 and AIEC strains. These genes included an excisionase, the *pduC* gene, and four CRISPR-associated genes.

Similarly, a new report comparing 14 AIEC strains isolated from IBD patients with another 37 non-IBD *E. coli* strains found that all IBD isolates contained three genomic islands, GI-18, GI-22, and GI-23, which were also present in LF82 and NRG857c strains. In addition, three genomic regions were found, RD2, RD8, and RD9, as well as the presence of T6SS genes. In the unique RD regions, there are four genes associated with AIEC, *insA*, *insB* (transposon-associated genes), integrase, and the vacuolating autotransporter toxin Vat, but there are other nine genes encoding uncharacterized proteins that could be important markers for AIEC strains (Desilets et al. 2016). Studies of the AIEC *vat* gene found that it was overrepresented in AIEC isolates (46 %) relative to non-AIEC isolates from CD patients (12 %), and 97 % of *E. coli* harboring the AIEC *vat* gene belonged to the B2 phylogenetic group (Gibold et al. 2015).

3 Role of Other *E. coli* in Dysbiosis and Homeostasis

3.1 Enterohemorrhagic E. coli and Dysbiosis

Enterohemorrhagic *E. coli* (EHEC) is one of the five enterovirulent *E. coli* that have direct interaction with human enterocytes and are the causative agent of hemorrhagic colitis and, in severe cases, hemolytic-uremic syndrome (HUS). Although

EHEC are pathogens as described in detail in Chap. 5 of this book, it has also been associated with microbiome dysbiosis. The main features of EHEC infection include a low infectious dose (between 50 and 100 CFU) and expression of Shiga toxins (Stx). EHEC causes a histopathological lesion called attaching and effacing (AE) lesion (reviewed in (Croxen et al. 2013)) and has been found to be firmly attached to the thick inner mucus layer of colonic cells unlike commensal *E. coli*, which are located in the outer, loose mucus layer facing the intestinal lumen. This bacterial localization between the outer mucus layer and colonic epithelium is thought to promote dysbiosis by EHEC (Pacheco and Sperandio 2015).

EHEC employs different strategies to achieve this regional colonization. These strategies include quorum-sensing (QS) signaling to regulate expression of its virulence genes. Further, EHEC produces a plasmid-encoded metalloprotease, StcE, which degrades intestinal mucins, which serve as a nutrient source and as a signal to trigger production of other virulence factors. Among the virulence factors that maximize EHEC proliferation are iron scavenging, chemotaxis, and motility (Croxen et al. 2013; Pifer and Sperandio 2014; Pacheco and Sperandio 2015).

The metabolic activity of commensal bacteria such as *Bacteroides thetaiotaomicron*, a predominant species of the ileal-cecal region of the gut, supports the proper growth and tissue tropism by EHEC strains. *B. thetaiotaomicron* generates different monosaccharides as end products of metabolism, providing nutrients for other commensals. Since EHEC lacks the enzymatic capacity to metabolize complex host-derived glycans, it consumes *B. thetaiotaomicron* metabolic by-products (Fig. 12.1). During intestinal colonization, commensal and pathogenic *E. coli* use similar carbon sources such as mucin for monosaccharides and disaccharides. This competition for carbon sources forms the basis of the "barrier effect" of commensal *E. coli* over enterovirulent strains.

However, EHEC metabolizes mucus-associated carbohydrates in vivo at a higher rate than microbiome-associated bacteria, including commensal *E. coli*, allowing EHEC to successfully compete for colonization of the mammalian intestine. Moreover, it has been shown that EHEC preferentially catabolize some specific carbon sources different for commensal *E. coli*, thereby avoiding competition for nutrients. It is known from in vivo experiments that EHEC is able to preferentially consume mannose, N-acetyl-glucosamine [GluNAc], N-acetyl neuraminic acid [Neu5Ac], and galactose, conferring a growth advantage to EHEC, at least in the bovine intestine (Bertin et al. 2013). Using a murine model of infection, it was found that the differential utilization of carbon sources by EHEC, e.g. galactose, hexuronates, mannose, and ribose, contributes to tissue tropism of this pathogen and no competition with commensal *E. coli*, which only use gluconate and N-acetyl-neuraminic acid as carbon sources (Fabich et al. 2008).

Another by-product of *B. thetaiotaomicron* metabolism is fucose, which is the key element that indirectly regulates transcription of EHEC virulence factors. Regulation occurs through activation of the FusKR two-component signaling system, which causes repression of the Locus for Enterocyte Effacement (LEE) Pathogenicity Island and fucose metabolism genes (Pacheco et al. 2012). Therefore, EHEC conserves energy by avoiding expression of virulence genes while in the

outer loose mucus layer and allowing commensal *E. coli* to use fucose for growth (Fig. 12.1) (Pacheco and Sperandio 2015; Vogt et al. 2015). Ler, the LEE's master regulator, is repressed when EHEC is growing in a fucose-rich environment. Therefore, nutrient modulation activity of the commensal microbiome dictates the outcome of the interaction of EHEC with IEC. In contrast, when EHEC penetrates into the deep mucus layer, where the normal microflora is absent, EHEC increases motility and the expression of Stx and AE lesion genes. This is mediated through additional two-component regulatory systems, QseBC and QseEF (extensively reviewed in Pacheco and Sperandio 2015; Vogt et al. 2015).

Ethanolamine is a product of epithelial cell turnover and an end-product of gut microbiome metabolism, which is abundant in the gastrointestinal tract. EHEC sense ethanolamine by EutR (ethanolamine receptor) and uses it as a nitrogen-source and signaling molecule. Ethanolamine triggers EHEC LEE and Stx expression and the QseC and QseE adrenergic sensors (Kendall et al. 2012; Pifer and Sperandio 2014; Pacheco and Sperandio 2015; Vogt et al. 2015). Moreover, three short-chain fatty acids (SCFA), acetate, propionate, and butyrate, are predominant in the colon, which is the site of EHEC tissue tropism. These SCFA promote EHEC expression of *iha* (encoding a non-fimbrial adhesin) (Fig. 12.1) and stimulate production and functionality of flagella (Herold et al. 2009; Tobe et al. 2011). Butyrate is more abundant in the colon than in the small intestine and it increases expression of LEE genes. In addition, it has been reported that a high-fiber diet increases butyrate levels and may increase susceptibility of animals to Stx, by promoting the expression of the toxin receptor globotriaosiylceramide (Nakanishi et al. 2009; Zumbrun et al. 2013). Succinate is another intermediary metabolite produced by the gut microbiome that promotes infection by EHEC via increasing type 3 secretion system (T3SS) expression.

Other evidence for the protective barrier effect of the gut microbiome on EHEC arises from studies using cell-free extracts from cultures of *B. thetaiotaomicron*. Sablet et al. reported that *B. thetaiotaomicron* secreted small peptides (<3 kDa) that prevented Stx2 expression, by inhibiting the EHEC SOS response mediated by RecA and thus the lytic induction of *stx* phages (de Sablet 2009). Disruption in the normal gut microbiome could therefore adversely affect its protective effect, thereby rendering the host more susceptible to EHEC infection and at greater risk for developing HUS.

3.2 E. coli Nissle 1917 and Homeostasis

As a result of several studies into the beneficial effect of the human microbiome, a group of specific bacterial species with health-promoting capabilities for treating dysbiosis have been selected. These organisms are called probiotics, which modulate IEC and dendritic cell function and induce a medium cellular response mediated by PAMPs recognition. One of them is *E. coli* Nissle 1917 (EcN), which is involved in maintaining homeostasis between host and its associated microbiome

(Nissle 1918). A. Nissle reported this antagonistic capacity of EcN against pathogenic bacteria, including diffuse adhering *E. coli* (DAEC) and uropathogenic *E. coli* (UPEC) strains, *Proteus vulgaris, Salmonella enteritidis, Shigella dysenteriae, Yersinia enterocolitica, Vibrio cholerae*, and *Candida albicans* (reviewed in (Sonnenborn and Schulze 2009)). Metabolically, EcN is a typical *E. coli* strain belonging to the O6:K5:H1 serotype. Clinical application of EcN includes the prevention of colonization and treatment of gastrointestinal infections by DAEC. Specifically, it has an inhibitory effect on adhesion and invasion of IEC by AIEC and, in neonates, is used as a prophylactic against colonization by enteropathogens (Boudeau et al. 2003; Sonnenborn and Schulze 2009; Lebeer et al. 2010; Pacheco and Sperandio 2015). EcN interacts with IEC using several surface molecules, for example, flagellin, fimbriae, and lipopolysaccharide (LPS) (Fig. 12.1) (Fischer et al. 2006; Schlee et al. 2007; Lasaro et al. 2009). In addition, EcN is able to synthesize several microcins, such as Microcin H47 and Microcin M, which explains its antagonist properties against other susceptible microorganisms (Sonnenborn and Schulze 2009).

EcN contributes to human health by enhancing the function of the intestinal epithelial barrier through expression of human β–defensin 2 (BD2 or DEFB4) by flagellin induction. This molecule prevents adherence and invasion of pathogens. The different outcome of the interaction between flagellin from commensal or probiotic organisms and flagellin from pathogens lies in the compartmentalization of TLR5, the amount of flagellin that can interact with TLR5, and the specific structural features of the flagellin monomers (Lebeer et al. 2010). EcN also expresses type 1 and F1C fimbriae. Both of these fimbriae are involved in biofilm formation and intestinal adherence. Although there is no direct evidence for the interaction of these fimbriae with host receptors, it is believed that EcN fimbriae bind TLR4 and specific co-receptors in a similar way as fimbriae from commensal and pathogenic *E. coli*. In addition, it has recently been reported that K5 cell wall-associated polysaccharide (CPS) of EcN is able to induce cytokine expression (immunomodulatory effect); however, the mechanism is still unknown. In pathogens, CPS are key virulence factors that act by impeding phagocytosis (Lebeer et al. 2010). On the other hand, LPS of EcN differs at the molecular level from all other LPS types of *E. coli*. For instance, the O6 polysaccharide side-chain is very short and it has only one single repeating-unit of the oligosaccharide. Furthermore, the acyl chains number (hexa instead of penta) and the length of lipid A and phosphorylation level and type of substitutions in the acyl chain are different. All these differences affect the outcome of LPS-TLR4 signaling and are discriminators between pathogens and commensals or probiotics (Sonnenborn and Schulze 2009; Lebeer et al. 2010).

Acetic acid, another by-product of EcN metabolism, is suggested to promote colonic motility in patients suffering from chronic constipation. In IBD patients, it has been shown that EcN stimulates IL-10 production by peripheral mononuclear cells. Moreover, EcN prevents the invasion of pathogens into enterocytes, enhances intestinal motility, and induces the synthesis of the tight junction proteins and human β–defensin. Interestingly, it has been shown that EcN inhibits enterocyte invasion by *Salmonella, Shigella,* enteroinvasive *E. coli, Listeria, Yersinia*, and

Legionella strains. The anti-invasive effect is not plasmid-encoded, but thought to be through a soluble EcN factor that is fimbriae-, microcins-, and contact-independent. It is suggested that this soluble factor somehow inhibits rearrangement of the host cell cytoskeleton by the enteropathogen (Sonnenborn and Schulze 2009; Lebeer et al. 2010).

4 Homeostasis vs. Dysbiosis, *E. coli* Studies in the Americas

Not many studies have focused their attention on the prevalence of IBD and the potential underlying causes in Latin America (Vargas 2010). Victoria et al. studied the incidence rates (existing cases/100,000 inhabitants/period) of IBD in a region of the state of São Paulo, Brazil, during the years 1986–2005. In the 2001–2005 period, the prevalence rate of total IBD (including CD and UC) in this geographical region of Brazil was 22.6, which represented an increase from the prevalence rate of 5.6 in the 1991–1995 period (Victoria et al. 2009). In another article, Yepes Barreto et al. reported a prevalence of 29 cases per 100,000 inhabitants in Cartagena, Colombia (Barreto et al. 2010). These reports revealed that, although the numbers are low, relative to the prevalence of IBD in regions where the highest rates have been reported (37.5–248.6 per 100,000 in North America for ulcerative colitis (UC) (Ananthakrishnan 2015)), IBD incidence and prevalence rates are constantly changing worldwide and could be particularly increasing in certain industrialized regions of the Americas.

What environmental and dietary factors alter homeostasis in subjects who develop IBD and how these changes alter the microbiome is still an area that has not been explored extensively in Latin America. However, two groups have performed a gut microbiome analysis in subjects residing in Colombia (Escobar et al. 2014) and Argentina (Carbonetto et al. 2016), which suggested that there are distinct regional differences in the gut microbiome. These studies can serve as a starting point for further investigations into whether differences in the microbiome composition, and therefore differences in the microenvironment of the gut, can be attributed to different IBD prevalence rates between Latin and North America. Additionally, other important questions remain unanswered, such as what are noticeable alterations that occur in microbiome composition in IBD patients in Latin America, and what is the role of *E. coli*, since several reports suggest these organisms are isolated in increased numbers from biopsies of IBD patients (Darfeuille-Michaud et al. 2004; Packey and Sartor 2009).

At a mechanistic level, studies in Latin America have explored the changes in gut homeostasis during IBD. Romero et al. explored some of the changes that occurred in the intestine of CD patients at the inflammatory level. In a sample of intestinal biopsies from the Federal University of São Paulo; Brazil, these investigators found that a larger percentage of inflammatory cells in the biopsies from CD patients expressed Cyclooxygenase-2 (COX-2) relative to healthy controls. The authors postulated that COX-2 expression could represent a mechanism underlying

the pathogenesis of inflammation observed in IBD (Romero et al. 2008). This finding is consistent with those of another study, showing that low doses of selective COX-2 inhibitors may contribute to gut barrier protection in intestinal inflammatory disorders (Short et al. 2013). Nevertheless, studies to identify the specific contribution of members of the microbiome, *E. coli* in particular, to the disruption of gut homeostasis are rare.

A novel study by De la Fuente et al. (2014) characterized a group of *E. coli* isolates lacking traditional virulence factors obtained from intestinal specimens from a cohort of IBD patients in Chile. In this study, the authors found that the numbers of intracellular *E. coli* were elevated in the samples obtained from IBD patients compared to healthy controls. Furthermore, it was found that an AIEC strain was predominant in isolates from CD patients. These characteristics, together with their ability to induce secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α by macrophages, make these intracellular *E. coli* potential contributors to the pathogenesis observed in IBD, where an inflammatory response causes the disruption of gut homeostasis. It is important to mention that in this study, *E. coli* HS from a healthy subject was also able to induce the secretion of IL-1β, IL-6, and TNF-α in macrophages. However, *E. coli* HS displayed significantly reduced intracellular survival compared to *E. coli* isolates from IBD patients. From this finding, it is possible to speculate that a contribution to inflammation and disruption of homeostasis in the gut by *E. coli* may occur if certain specific scenarios are presented, such as defective intracellular clearance of *E. coli* with specific variants of pattern-recognition receptors (PRR).

E. coli can also benefit from alterations observed in IBD such as increased intestinal permeability in the gut. Espinoza et al. (2014) reported constant episodes of bacteremia (by *E. coli* in a patient with CD), possibly due to alterations in intestinal permeability. Thomazini and Samegima (2011) also studied the characteristics of a group of *E. coli* isolates from IBD patients in Southeast Brazil. Consistent with previous reports (Lapaquette and Darfeuille-Michaud 2010), the biopsies of these IBD patients (UC and CD) contained higher numbers of mucosa-associated *E. coli* relative to control subjects. In this study, the authors also found that no particular clone or clonal group of *E. coli* was associated with CD or UC and, additionally, a group of *E. coli* isolates from UC subjects (39.1 %) was positive for some traditional virulence factors described in other enterovirulent *E. coli*. The high degree of heterogeneity of *E. coli* isolates obtained from IBD patients makes it difficult to determine whether commensal *E. coli* or strains with an AIEC phenotype are favored during the dysbiosis that tends to be observed in IBD. While *E. coli* can be associated with a contribution to inflammation and pathologies such as IBD, it is important to mention that certain isolates can show a protective effect. Souza et al.(Souza et al. 2016) found that the EcN strain reduced the expression of pro-inflammatory cytokine IL-1β and the histopathological signs of colitis in a murine model of colitis.

In North America, Sepehri et al. (2011) characterized *E. coli* isolates from IBD biopsies collected in Canada. Consistent with previous observations, *E. coli* isolated from IBD biopsies were able to survive intracellularly within macrophages more

efficiently than isolates from the respective controls. The authors described the presence of the AIEC phenotype among the IBD isolates. These AIEC isolates shared genetic similarities with extra-intestinal *E. coli,* but, at the same time, unique gene sequences were present within this group, suggesting a potential specialization of a group of *E. coli* in an IBD microenvironment. Desilets et al. (2016) further explored this collection of strains by whole-genome sequence-based analysis and found that these AIEC isolates, initially characterized phenotypically, were phylogenetically related and can be differentiated from commensal *E. coli* at three specific genomic regions. Both studies highlight the importance of understanding IBD-associated *E. coli* (at the phenotypic and genotypic level) in order to gain further insight into the transition between commensalism and pathogenicity in certain *E. coli* isolates; especially under conditions where gut homeostasis has been disrupted.

To summarize, some studies into the role of *E. coli* in gastrointestinal disorders, such as IBD, where dysbiosis is a hallmark have focused on screening the microbiome populations in different geographical regions in both Latin and North America (Vargas 2010; Escobar et al. 2014; Carbonetto et al. 2016). Others aimed to describe the potential association between *E. coli* and inflammation (la Fuente et al. 2014) and the characteristics that might make certain *E. coli* isolates thrive or cause a disruption in gut homeostasis (Sepehri et al. 2011; Desilets et al. 2016). Nevertheless, further studies are required to determine whether IBD-associated *E. coli* isolates can cause pathogenesis beyond the clinical associations observed. Further exploration of IBD-associated *E. coli* in animal models as demonstrated by Small et al. (2013) would be an ideal initial step in order to generate further consensus whether these isolates present specific traits that can disrupt homeostasis in the host gut via inflammation or if it is more a case of specific commensal *E. coli* populations being favored by disruptions of certain host microbiome homeostasis, supporting their survival. The studies also emphasize the critical environmental and genetic factors that affect homeostasis and dictate the relationship between the host species and its microbiome. Latin America is a specifically fertile region to further explore the characteristics of the microbiome and how it is affected by inflammatory intestinal disorders.

References

Ananthakrishnan AN (2015) Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol 12:205–217

Barnich N (2007) CEACAM6 acts as a receptor for adherent-invasive *E. coli*, supporting ileal mucosa colonization in Crohn disease. J Clin Invest 117:1566–1574

Barnich N, Boudeau J, Claret L, Darfeuille-Michaud A (2003) Regulatory and functional cooperation of flagella and type 1 pili in adhesive and invasive abilities of AIEC strain LF82 isolated from a patient with Crohn's disease. Mol Microbiol 48:781–794

Barreto I, Carmona R, Díaz F (2010) Prevalencia y características demográficas de la enfermedad inflamatoria intestinal en Cartagena, Colombia. Rev Colomb Gastroenterol 2:107–111

Baumler AJ, Tsolis RM, Heffron F (1996) The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. Proc Natl Acad Sci 93:279–283

Bertin Y, Chaucheyras Durand F, Robbe Masselot C et al (2013) Carbohydrate utilization by enterohaemorrhagic *Escherichia coli* O157:H7 in bovine intestinal content. Environ Microbiol 15:610–622

Boudeau J, Glasser A-L, Julien S et al (2003) Inhibitory effect of probiotic E*scherichia coli* strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent-invasive E. coli strains isolated from patients with Crohn's disease. Aliment Pharmacol Ther 18:45–56

Bringer M-A, Glasser A-L, Tung C-H et al (2006) The Crohn's disease-associated adherent-invasive *Escherichia coli* strain LF82 replicates in mature phagolysosomes within J774 macrophages. Cell Microbiol 8:471–484

Carbonetto B, Fabbro MC, Sciara M et al (2016) Human microbiota of the argentine population—a pilot study. Front Microbiol 7:51

Carvalho FA, Barnich N, Sivignon A et al (2009) Crohn's disease adherent-invasive *Escherichia coli* colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. J Exp Med 206:2179–2189

Chassaing B, Rolhion N, de Vallee A et al (2011) Crohn disease–associated adherent-invasive *E. coli* bacteria target mouse and human Peyer's patches via long polar fimbriae. J Clin Invest 121:966–975

Cieza RJ, Hu J, Ross BN et al (2015) The IbeA invasin of adherent-invasive *Escherichia coli* mediates interaction with intestinal epithelia and macrophages. Infect Immun 83:1904–1918

Consortium THMP (2012) Structure, function and diversity of the healthy human microbiome. Nature 486:207–214

Cooney R, Baker J, Brain O et al (2010) NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med 16:90–97

Croxen MA, Law RJ, Scholz R et al (2013) Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26:822–880

Darfeuille-Michaud A (2002) Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. Int J Med Microbiol 292:185–193

Darfeuille-Michaud A, Neut C, Barnich N et al (1998) Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. Gastroenterology 115:1405–1413

Darfeuille-Michaud A, Boudeau J, Bulois P et al (2004) High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. Gastroenterology 127:412–421

de Lange KM, Barrett JC (2015) Understanding inflammatory bowel disease via immunogenetics. J Autoimmun 64:91–100

de Sablet T (2009) Human microbiota-secreted factors inhibit shiga toxin synthesis by enterohemorrhagic Escherichia coli O157:H7. Infect Immun 77:783–790

Desilets M, Deng X, Rao C et al (2016) Genome-based definition of an inflammatory bowel disease-associated adherent-invasive *Escherichia coli* pathovar. Inflamm Bowel Dis 22:1–12

Dreux N, Denizot J, Martinez-Medina M et al (2013) Point mutations in FimH adhesin of Crohn's disease-associated adherent-invasive *Escherichia coli* enhance intestinal inflammatory response. PLoS Pathog 9, e1003141

Eaves-Pyles T, Allen CA, Taormina J et al (2008) *Escherichia coli* isolated from a Crohn's disease patient adheres, invades, and induces inflammatory responses in polarized intestinal epithelial cells. Int J Med Microbiol 298:397–409

Economou M, Trikalinos TA, Loizou KT et al (2004) Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a meta-analysis. Am J Gastroenterol 99:2393–2404

Escobar JS, Klotz B, Valdes BE, Agudelo GM (2014) The gut microbiota of Colombians differs from that of Americans, Europeans and Asians. BMC Microbiol 14:1

Espinoza R, Quera R, Meyer L, Rivera D (2014) Trasplante de microbiota fecal: primer caso reportado en Chile y revisión de la literatura. Rev Chilena Infectol 31:477–482

Fabich AJ, Jones SA, Chowdhury FZ et al (2008) Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. Infect Immun 76:1143–1152

Fischer H, Yamamoto M, Akira S et al (2006) Mechanism of pathogen-specific TLR4 activation in the mucosa: fimbriae, recognition receptors and adaptor protein selection. Eur J Immunol 36:267–277

Frank DN, St Amand AL, Feldman RA et al (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci 104:13780–13785

Gibold L, Garenaux E, Dalmasso G et al (2015) The Vat-AIEC protease promotes crossing of the intestinal mucus layer by Crohn's disease-associated *Escherichia coli*. Cell Microbiol 5:617–631

Hase K, Kawano K, Nochi T et al (2009) Uptake through glycoprotein 2 of FimH+ bacteria by M cells initiates mucosal immune response. Nature 462:226–230

Herold S, Paton JC, Srimanote P, Paton AW (2009) Differential effects of short-chain fatty acids and iron on expression of iha in Shiga-toxigenic *Escherichia coli*. Microbiology 155:3554–3563

Huang SH, Wan ZS, Chen YH et al (2001) Further characterization of *Escherichia coli* brain microvascular endothelial cell invasion gene *ibeA* by deletion, complementation, and protein expression. J Infect Dis 183:1071–1078

Janka Babickova RG (2015) Pathological and therapeutic interactions between bacteriophages, microbes and the host in inflammatory bowel disease. World J Gastroenterol 21:11321–11330

Jensen SR, Nielsen OH, Brix S (2011) Are NOD2 polymorphisms linked to a specific disease endophenotype of Crohn's disease? Inflamm Bowel Dis 17:2392–2401

Keely S, Walker MM, Marks E, Talley NJ (2015) Immune dysregulation in the functional gastrointestinal disorders. Eur J Clin Invest 45:1350–1359

Kendall MM, Gruber CC, Parker CT, Sperandio V (2012) Ethanolamine controls expression of genes encoding components involved in interkingdom signaling and virulence in enterohemorrhagic *Escherichia coli* O157:H7. mBio 3:e00050–12

la Fuente DM, Franchi L, Araya D et al (2014) *Escherichia coli* isolates from inflammatory bowel diseases patients survive in macrophages and activate NLRP3 inflammasome. Int J Med Microbiol 304:384–392

Lapaquette P, Darfeuille-Michaud A (2010) Abnormalities in the handling of intracellular bacteria in Crohn's disease. J Clin Gastroenterol 44:S26–S29

Lasaro MA, Salinger N, Zhang J et al (2009) F1C fimbriae play an important role in biofilm formation and intestinal colonization by the *Escherichia coli* commensal strain Nissle 1917. Appl Environ Microbiol 75:246–251

Lebeer S, Vanderleyden J, De Keersmaecker SCJ (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8(3):171–184

Lederberg J, Mccray AT (2001) 'Ome Sweet' omics—a genealogical treasury of words. Scientist 15:8

Lloyd SJ, Ritchie JM, Rojas-Lopez M et al (2012) A double, long polar fimbria mutant of *Escherichia coli* O157:H7 expresses curli and exhibits reduced *in vivo* colonization. Infect Immun 80:914–920

Martinez-Medina M, Garcia-Gil LJ (2014) *Escherichia coli* in chronic inflammatory bowel diseases: an update on adherent invasive Escherichia coli pathogenicity. World J Gastrointest Pathophysiol 5:213–227

Miquel S, Peyretaillade E, Claret L et al (2010) Complete genome sequence of Crohn's disease-associated adherent-invasive *E. coli* strain LF82. PLoS One 5:e12714

Mizoguchi A, Takeuchi T, Himuro H et al (2016) Genetically engineered mouse models for studying inflammatory bowel disease. J Pathol 238:205–219

Nakanishi N, Tashiro K, Kuhara S et al (2009) Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli*. Microbiology 155:521–530

Nash JH, Villegas A, Kropinski AM et al (2010) Genome sequence of adherent-invasive Escherichia coli and comparative genomic analysis with other *E. coli* pathotypes. BMC Genomics 11:667

Nguyen HTT, Dalmasso G, Muller S et al (2014) Crohn's disease-associated adherent invasive *Escherichia coli* modulate levels of microRNAs in intestinal epithelial cells to reduce autophagy. Gastroenterology 146:508–519

Nilsson LM, Thomas WE, Sokurenko EV, Vogel V (2006) Elevated shear stress protects *Escherichia coli* cells adhering to surfaces via catch bonds from detachment by soluble inhibitors. Appl Environ Microbiol 72:3005–3010

Nissle A (1918) Die antagonistische Behandlung chronischer Darmstörungen mit Colibakterien. Med Klin 2:29–33

O'Brien CL, Pavli P, Gordon DM, Allison GE (2014) Detection of bacterial DNA in lymph nodes of Crohn's disease patients using high throughput sequencing. Gut 63:1596–1606

Pacheco AR, Sperandio V (2015) Enteric pathogens exploit the microbiota-generated nutritional environment of the gut. Microbiol Spectr 3(3)

Pacheco AR, Curtis MM, Ritchie JM et al (2012) Fucose sensing regulates bacterial intestinal colonization. Nature 492:113–117

Packey CD, Sartor RB (2009) Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Curr Opin Infect Dis 22:292–301

Pifer R, Sperandio V (2014) The interplay between the microbiota and enterohemorrhagic *Escherichia coli*. Microbiol Spectr 2(5)

Rolhion N, Barnich N, Claret L, Darfeuille-Michaud A (2005) Strong decrease in invasive ability and outer membrane vesicle release in Crohn's disease-associated adherent-invasive *Escherichia coli* strain LF82 with the *yfgL* gene deleted. J Bacteriol 187:2286–2296

Rolhion N, Barnich N, Bringer M-A et al (2010) Abnormally expressed ER stress response chaperone Gp96 in CD favors adherent-invasive *Escherichia coli* invasion. Gut 59:1355–1362

Romero M, Artigiani R, Costa H et al (2008) Evaluation of the immunoexpression of COX-1, COX-2 and p53 in Crohn's disease. Arq Gastroenterol 45:295–300

Schlee M, Wehkamp J, Altenhoefer A et al (2007) Induction of human beta-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. Infect Immun 75:2399–2407

Sepehri S, Khafipour E, Bernstein CN et al (2011) Characterization of *Escherichia coli* isolated from gut biopsies of newly diagnosed patients with inflammatory bowel disease. Inflamm Bowel Dis 17:1451–1463

Short SS, Wang J, Castle SL et al (2013) Low doses of celecoxib attenuate gut barrier failure during experimental peritonitis. Lab Invest 93:1265–1275

Small C-LN, Reid-Yu SA, McPhee JB, Coombes BK (2013) Persistent infection with Crohn's disease-associated adherent-invasive *Escherichia coli* leads to chronic inflammation and intestinal fibrosis. Nat Commun 4:1957

Sokol H, Seksik P, Rigottier-Gois L et al (2006) Specificities of the fecal microbiota in inflammatory bowel disease. Inflamm Bowel Dis 12:106–111

Sonnenborn U, Schulze J (2009) The non-pathogenic *Escherichia coli* strain Nissle 1917—features of a versatile probiotic. Microb Ecol Health Dis 21:122–158

Souza ÉL, Elian SD, Paula LM et al (2016) *Escherichia coli* strain Nissle 1917 ameliorates experimental colitis by modulating intestinal permeability, the inflammatory response and clinical signs in a faecal transplantation model. J Med Microbiol 65:201–210

Thomazini CM, Samegima D (2011) High prevalence of aggregative adherent *Escherichia coli* strains in the mucosa-associated microbiota of patients with inflammatory bowel diseases. Intl J Med Microbiol 6:475–479

Tobe T, Nakanishi N, Sugimoto N (2011) Activation of motility by sensing short-chain fatty acids via two steps in a flagellar gene regulatory cascade in enterohemorrhagic *Escherichia coli*. Infect Immun 79:1016–1024

Torres AG, Girón JA, Perna NT et al (2002) Identification and characterization of *lpfABCC'DE*, a fimbrial operon of enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 70:5416–5427

Torres AG, Kanack KJ, Tutt CB et al (2004) Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. FEMS Microbiol Lett 238:333–344

Travassos LH, Carneiro LAM, Ramjeet M et al (2010) Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol 11:55–62

Vargas RD (2010) Epidemiología de la enfermedad inflamatoria intestinal: ¿por qué las diferencias entre Norteamérica y Latinoamérica? Rev Colomb Gastroenterol 25(2):103–105

Vazeille E, Chassaing B, Buisson A et al (2016) GipA factor supports colonization of Peyer's patches by Cohn's disease-associated *Escherichia coli*. Inflamm Bowel Dis 22:68–81

Victoria CR, Sassak LY, Nunes HR de C (2009) Incidence and prevalence rates of inflammatory bowel diseases, in Midwestern of São Paulo State, Brazil. Arq Gastroenterol 46:20–25

Vogt SL, Pena-Diaz J, Finlay BB (2015) Chemical communication in the gut: Effects of microbiota-generated metabolites on gastrointestinal bacterial pathogens. Anaerobe 34:106–115

Yazdanyar S, Weischer M, Nordestgaard BG (2009) Genotyping for NOD2 genetic variants and Crohn disease: a meta-analysis. Clin Chem 55:1950–1957

Zhang Y, Rowehl L, Krumsiek JM et al (2015) Identification of candidate adherent-invasive *E. coli* signature transcripts by genomic/transcriptomic analysis. PLoS One 10, e0130902

Zumbrun SD, Melton-Celsa AR, Smith MA et al (2013) Dietary choice affects Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 colonization and disease. Proc Natl Acad Sci U S A 110:E2126–E2133

Chapter 13
Antibiotic Resistance in *Escherichia coli*

Theresa J. Ochoa and Oscar G. Gómez-Duarte

Summary The Latin American region faces a significant challenge with high levels of antimicrobial resistance among important Gram-negative organisms, including *E. coli*. In recent years, extended-spectrum β-lactamases (ESBLs) have increased in type and frequency, carbapenemases have emerged, and multidrug-resistant *E. coli* has spread across the American continent. It is also important to recognize the worldwide spread of the *E. coli* ST131 clones and subclones with fluoroquinolone and ESBL resistance, and in some instances, resistant to carbapemens and aminoglycosides. Resistance in *E. coli* is generated by positive selection through single point mutations, which is often the case for fluoroquinolone resistance, or by acquisition of mobile genetic elements, which has been the case for broad spectrum penicillins and third-generation cephalosporins. Successful resistance clones may continue to evolve into unique subclones and spread worldwide as observed with ST131 subclones. The frequency of *E. coli* antimicrobial resistance varies by geographic area and it is not possible to establish universal guidelines on the use of antibiotics. It is critical that every local healthcare institution establishes an antimicrobial stewardship program to promote the proper use of

T.J. Ochoa (✉)
Departamento de Pediatría, Instituto de Medicina Tropical,
Universidad Peruana Cayetano Heredia, Lima, Peru

Center for Infectious Diseases, School of Public Health,
University of Texas Health Science Center, Houston, TX, USA
e-mail: Theresa.J.Ochoa@uth.tmc.edu

O.G. Gómez-Duarte
Division of Pediatric Infectious Diseases, Vanderbilt University
School of Medicine, Nashville, TN, USA
e-mail: oscar.gomez@Vanderbilt.Edu

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_13

antibiotics, restricting their use, audit clinical use in real-time, and provide feedback to the treating clinician. Implementation of antimicrobial stewardship programs is the best way to optimize clinical outcomes, decrease further antibiotics resistance, and limit healthcare cost associated with management of multidrug-resistant *E. coli* infections.

1 Introduction

Escherichia coli are part of the normal flora in the intestine in humans and animals. Nevertheless, they are also the most frequent cause of community and hospital-acquired urinary tract infections, bloodstream infection at all ages, meningitis in neonates, and one of the leading agents of food-borne infections worldwide. Extra-intestinal *E. coli* infections usually originate by bacterial translocation from the gut to sterile organ-systems (auto infection). Transmission of resistant *E. coli* may also occur from animals, either directly (zoonosis) or through the food chain (food-borne). Antimicrobial-resistant *E. coli* has been detected in all parts of the world and it is one of the greatest challenges to public health today in the Americas. Because antimicrobial resistance is a natural phenomenon of positive selection, misuse of antimicrobials, inadequate or inexistent programs for infection prevention and control, poor-quality medications, weak laboratory capacity, inadequate surveillance, and insufficient regulation for the use of antimicrobial medications are crucial factors that contribute to a worldwide spread (World Health Organization 2014). In this chapter, we will review tthe mechanisms of antibiotic resistance in *E. coli*, advances in the study of antibiotic resistance in the American continent, and the link between antimicrobial resistance with *E. coli* pathogenesis and clinical outcomes.

2 General Concepts About Mechanisms of Antibiotic Resistance in *E. coli*

Bacteria can be intrinsically resistant to certain antibiotics, but can also acquire resistance to antibiotics via mutations in chromosomal genes and by acquisition of antimicrobial resistance genes by horizontal transfer through episomal DNA (transposons, plasmids, and bacteriophages). Genotypic-based methods hold promise for the rapid and accurate detection or confirmation of antimicrobial resistance. Unfortunately, these methods only detect resistance, not susceptibility; consequently, phenotypic methods in clinical practice will continue to guide antibiotic therapy, even though turnaround times remain long (Blanco et al. 1996; Louie and Cockerill 2001).

2.1 Mechanism of E. coli Antimicrobial Resistance

The main antimicrobial resistance mechanisms are: (1) *Prevention of access to target*, by reduced permeability or increased efflux. Gram-negative bacteria are intrinsically less permeable to many antibiotics as their outer membrane forms a permeability barrier. For example, in *Enterobacteriaceae*, reductions in porin expression significantly contribute to resistance to newer drugs such as carbapenems and cephalosporins. On the other hand, bacterial efflux pumps actively transport many antibiotics out of the cell and are major contributors to the intrinsic resistance of Gram-negative bacteria to many of the drugs. Efflux pumps are divided in families, the most common ones include resistance nodulation division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), ATP-binding cassette (ABC), and small multidrug resistance (SMR), among others. When overexpressed, efflux pumps can also confer high levels of resistance to previously clinically useful antibiotics. (2) *Changes in antibiotic targets*. Most antibiotics specifically bind to bacterial targets with high affinity, thus preventing the normal activity of the target. Changes to the target structure that prevent efficient antibiotic binding leave the target intact to conduct normal function, and simultaneously, confer bacterial resistance to that antibiotic or class of antibiotic. In recent years, protection of targets has been found to be a clinically relevant mechanism of resistance for several important antibiotics; for example, the erythromycin ribosome methylase (*erm*) family of genes methylates 16S rRNA and alter the drug-binding. (3) *Direct modification of antibiotics*. Bacteria can destroy or modify antibiotics by a variety of chemical reactions, being antibiotic hydrolysis, chemical group transfer, or addition of a chemical group to vulnerable sites on an antibiotic molecule, the most common reactions. Inhibition by hydrolysis is the major mechanism of antibiotic resistance, which has been relevant since antibiotics were used for the first time and shortly after with the discovery of penicillinase (β-lactamase) in 1940 (Blair et al. 2015).

Resistance in *E. coli* readily develops either through single point mutations of antibiotic target genes, being fluoroquinolone resistance a classic example, or by acquisition of mobile genetic elements, as it has been the case for broad spectrum penicillins (e.g. ampicillin or amoxicillin) and third-generation cephalosporins resistance. The main antimicrobial resistance mechanisms of *E. coli* are summarized on Table 13.1.

2.2 β-Lactam Resistance

The most common mechanism of β-lactam resistance among Gram negative bacteria is β-lactamase production (Curello and MacDougall 2014). There are more than 1300 β-lactamases described to date, making them one of the most numerous natural enzyme families (Bush 2013a). Molecular classes A, B, C, and D define β-lactamases according to amino acid sequence and conserved motifs. In contrast, the functional classification, divided in groups 1, 2, and 3, is based on substrate and

Table 13.1 Main mechanisms of antimicrobial resistance studied in *E. coli*

Antibiotic family	Mechanism of antimicrobial resistance	Involved genes
β-lactams	β-lactamases: enzymes that can break the antibiotic structure by hydrolysis of the amide bond in the β-lactam nucleus	Genes codifying β-lactamases: bla_{TEM}, bla_{SHV}, bla_{CARB}, bla_{OXA}, $bla_{CTX\text{-}M}$, and bla_{GES}
Quinolones	Single mutations that alter the antibiotic target enzyme	Mutations in *gyrA* (gene encoding a subunit of the DNA gyrase) and *parC* (gene encoding a subunit of the topoisomerase IV)
	Alteration of permeation mechanisms (efflux pumps and diffusion channels)	AcrAB-like
	Antimicrobial resistance genes transported by plasmids	Family of *qnr* genes (A, B, C, D, S) that codify Qnr proteins that interfere link of the antibiotic with its target
		Gene that codifies an enzyme capable of inactivating fluoroquinolones: variant of cr of the acetyltransferase 6' (*aac* (6′)-lb-cr)
Tetracycline	Specific efflux pumps	*tetA* and *tetB* codify efflux systems
Chloramphenicol	Acetylation-mediated enzymatic inactivation	*cat* gene confers high-level resistance by coding chloramphenicol acetyltransferase
	Reduced membrane permeability	*florR* and *cmlA* genes
Trimethoprim-Sulfamethoxazole	Mutation of target enzymes	*sul1* and *sul2* genes: sulfamethoxazole resistance; *dfr* genes: trimethoprim resistance

Note: Modified from Mosquito et al. (2011)

inhibitor profiles in a way that can be correlated with phenotype in clinical isolates (Bush and Jacoby 2010; Bush 2013b). The most common families of β-lactamases are bla_{TEM}, bla_{SHV}, bla_{CARB}, bla_{OXA}, $bla_{CTX\text{-}M}$, and bla_{GES}. The first two (TEM and SHV) belong to the group 2b, which are penicillinases inhibited by clavulanic acid; CARB belongs to 2c, and hydrolyze carbenicillin; OXA, belongs to 2d and hydrolyze cloxacillin; CTX are broad spectrum and affect the activity of third- and fourth-generation cephalosporins; GES hydrolyze ceftazidime.

Resistance to third- and fourth-generation cephalosporins is mainly conferred by enzymes known as extended spectrum β-lactamases (ESBLs); these enzymes destroy many β-lactam antibacterial drugs. ESBLs genes are transmissible between bacteria of the same species and also between different bacterial species. *E. coli* strains that have ESBL are generally also resistant to several other antibacterial drugs. A recently emerging threat is carbapenem resistance in *E. coli*, mediated by metallo-β-lactamases, which confers resistance to virtually all available β-lactam antibacterial drugs.

2.3 Quinolone Resistance

The mechanism of action of quinolones is the inhibition of the topoisomerases type II (DNA gyrase and topoisomerase IV). Fluoroquinolone resistance is predominantly mediated by mutations in chromosomal genes and to a less degree by the presence of conjugative or non-conjugative plasmids carrying the quinolone resistance gene (*qnr*) or other genes. The main mechanisms include mutations in *gyrA*, the gene encoding a subunit of the DNA gyrase, and in *parC*, the gene encoding a subunit of the topoisomerase IV. In the last decade, several types of plasmid-mediated quinolone resistance (PMQR) genes have been described and reported worldwide, especially in *Enterobacteriaceae* (Strahilevitz et al. 2009). The PMQR genes include (a) five *qnr* families (*qnrA1–7*, *qnrB1–47*, *qnrC*, *qnrD,* and *qnrS1–58*), encoding small proteins that protect type II DNA topoisomerases from quinolones; (b) *aac* (6′)-Ib-cr, encoding a modified aminoglycoside acetyltransferase also active on certain fluoroquinolones; and (c) *qepA* and *oqxAB*, encoding quinolone-specific and multidrug efflux systems, respectively. The PMQR genes by themselves are able to confer only a moderate reduction in quinolone susceptibility, but they have been advocated as promoting the emergence of chromosomal mutations leading to resistance levels of clinical significance (Strahilevitz et al. 2009).

2.4 Antibiotic Use in Animals and Antibiotic Resistance

Feeding antibiotics to farm animals is used extensively worldwide for treatment, prevention, growth promotion, and ultimately to increase livestock industry profit. Antibiotics, in fact, are designated growth factors in the animal industry, and they have been used in Europe for more than 50 years. Antibiotic use in animals is associated with increased frequency of multidrug resistance organisms in animal gut, animal feces, animal environment, food products, and consequently human gut. The link between antibiotic resistance transmission to humans resulted in legislation change that limited its use in 2006 (Castanon 2007).

Spread of multidrug-resistant organisms from animals to the environment and eventually to humans may occur by multiple mechanisms. Antibiotic treatment of animals in a stable influences the pattern of intestinal *E. coli* resistance and commensal microbiota overall, not only in treated animals but also in untreated animals (Scherz et al. 2014; Beyer et al. 2015). It was shown that excessive use of animal manure on farms is associated with spread of antibiotic resistance gene to soil bacteria possibly by horizontal transfer of antibiotics resistance genes (Ghosh and LaPara 2007). One study in pigs showed piggery reservoirs for β-lactamases. It also showed that β-lactamases TEM-52 and CTX-M-1/-32 spread to piggeries was associated with plasmids and clone previously identified in humans and animals (Scherz et al. 2014).

3 Recent Advances on Multidrug Resistance *E. coli* Research

Worldwide spread of the multidrug-resistant *E. coli* sequence type 131 (ST131) clone threatens patients infected with these isolates and subjects colonized and at risk for infection. International public health authorities are concerned with the increased number of bacterial strains with high antimicrobial-resistant levels to commonly used antibiotics that are isolated from healthcare centers and from the community. Extended spectrum β-lactamases (ESBLs), often associated with resistance to aminoglycosides and fluoroquinolones, are characteristic of *E. coli* ST131, a clone that emerged in community-associated extra-intestinal *E. coli* infections (Giuffrè et al. 2013). The *E. coli* ST131 clinical isolates are associated with an increased number of extra-intestinal infections that may be particularly difficult to treat, prompt to recurrences, and that represent a significant antibiotic treatment challenge to clinicians worldwide (Banerjee and Johnson 2014).

3.1 Clinical Studies

The *E. coli* ST131-H30 subclone has emerged rapidly to become the leading antibiotic-resistant *E. coli* strain worldwide. Sequence type 131 refers to the number classification given to this clonal group of *E. coli* strains based on the multilocus sequencing typing system described by Achtman and colleagues (http://mlst.warwick.ac.uk/mlst/) (Wirth et al. 2006). H30 refers to a FimH type I fimbrial tip adhesin allele. Hyper-virulence, multi-drug resistance, and opportunistic infections have been proposed as explanations for its epidemic success. Johnson and collaborators assessed 1133 *E. coli* clinical isolates from five US medical centers (2010–2011) for H30 genotype with respect to host characteristics, clinical presentations, management, and outcomes. The H30 ($n = 107$) isolates were associated with hosts who were older, male, locally and systemically compromised, and healthcare and antibiotic-exposed. With multivariable adjustment for host factors, H30 was strongly associated with clinical persistence (odds ratio [OR] 3.47; 95 % confidence interval [CI] 1.89–6.37), microbiological persistence (OR 4.46; CI 2.38–8.38), subsequent hospital admission (OR 2.68; CI 1.35–5.33), and subsequent new infections (OR 1.73; CI 1.01–3.00) (Johnson et al. 2016).

Clinical evaluation of bloodstream infection caused by *E. coli* ST131 isolated from patients at 13 hospitals in Korea after multivariate analysis revealed that older age (OR 5.39, 95 % CI 1.22–23.89; $p = 0.027$) and nosocomial infection (OR 4.81, 95 % CI 1.15–20.15; $p = 0.032$) were independent risk factors for 30-day mortality (Cho et al. 2015). Urinary tract infections, in which *E. coli* ST131 was the etiologic agent, predicted antibiotic treatment failure. In a different study, multivariate analysis performed among quinolone-resistant isolates indicated that treatment failure was three times more common among the patients infected with the ST131 clone (OR 3; 95 % CI 1.27–7.4; $p = 0.012$) (Can et al. 2015). A similar study done in adults with *E. coli* isolated from predominantly urine samples revealed that ST131 was the

predominant antimicrobial-resistant clone associated with elderly host and with persistent or recurrent infections (Banerjee et al. 2013a).

In addition to bloodstream infection and urinary tract infections, there are multiple reports of different infection conditions as well as medical procedures complicated with *E. coli* ST131 infections. These included severe soft tissue infections with a confirmed multi-resistance pattern (Buchanan et al. 2014), infections following prostate biopsies, including bacteremia and prostate abscesses, among others (Suwantarat et al. 2014).

To understand the dimension of the *E. coli* ST131 spread, studies have evaluated the degree of gut colonization by ST131 clone among residents, patients, and healthcare workers at long-term rehabilitation facilities. Long-term rehabilitation facilities and nursing home residents were found to be colonized by *E. coli* ST131 ESBL-resistant and even carbapenem-resistant strain, some of which resulted in extra-intestinal infections (Valenza et al. 2015; Piazza et al. 2016). Person-to-person was believed to be one of transmission mechanisms involved among nursing home residents. Studies in US long-term care facilities found that multivariate predictors of ST131 clone colonization included inability of sign consent (OR 4.16 [$p=0.005$]), decubitus ulcer (OR 4.87 [$p=0.04$]), and fecal incontinence (OR 2.59 [$p=0.06$]), indicating that residents of these facilities with increased nursing care needs are at increasing risk for ST131 colonization (Burgess et al. 2015). Widespread of *E. coli* ST131 among ambulatory and inpatient adults confirms ST131 colonization in both healthy subject in the community and healthcare facility patients (Pietsch et al. 2015). In addition to adults, studies in children, although limited, also demonstrate the effect of ST131 in colonization and infection. Across neonatal facilities in England, *E. coli* ST131 was identified in stools of neonatal patients. It was not possible to determine if intestinal colonization was a result of vertical transmission from colonized mothers versus horizontal transmission from hospital personnel or medical equipment (Millar et al. 2016).

Multiple risk factors may also contribute to ST131 colonization, infection, and spread. One of these factors is international travel. A case-control study of US cases of positive ESBL *E. coli* infections and controls positive for non-ESBL *E. coli* infections found that risk factors for ESBL-producing *E. coli* infection included travel to India (OR 14.40; CI 2.92–70.95]), ciprofloxacin use (OR 3.92 [95 %, CI 1.90–8.1]), and age (OR 1.04 [95 %, CI 1.02–1.06]). Case isolates exhibited high prevalence of ST131 with CTX-M-15 beta-lactamase and *aac* (6′)-*Ib-cr* resistance genes (Banerjee et al. 2013b).

The dramatic spread of *E. coli* ST131 clone across the world may be facilitated not only by their ability to colonize healthy human hosts, but also domesticated animal or even wild animals. Studies in Australia showed that *E. coli* ST131 among humans and animals had identical patterns of antimicrobial resistance and similar patterns of chromosomal fluoroquinolone resistance mutations, indicating that these strains were exchanged possibly by the oral-fecal route (Platell et al. 2011). Broiler chickens with *E. coli* septicemia were positive for *E. coli* ST131 O25:H4 in Brazil and it is believed that healthy animal may also be colonized (Barbieri et al. 2015). *E. coli* ST131 colonization of camels was reported in Saudi Arabia (Fadlelmula et al. 2016) and *E. coli* ST131 was identified in feces from wild rodents in district 18 from

Hong Kong. These ST131 clones carried CTX-M genes and they were multidrug-resistant (Ho et al. 2015). Dissemination of multidrug-resistant *E. coli* ST131 by waste water in the natural environment is an additional factor believed to contribute to the spread of this clone across the land. This mechanism of dissemination was demonstrated in United Kingdom's natural environments such as rivers, lakes, and soil, and it's considered a threat to human and animal health (Amos et al. 2014).

3.2 Phylogenetic and Epidemiology

E. coli ST131, an important human pathogen extensively spread worldwide, is responsible for the rapid increase in antimicrobial resistance to multiple antibiotic classes. Two important genotypes associated with this multi-resistant clone are the presence of the CTX-M-15 gene-encoding ESBL and the type 1 fimbrial adhesin *fimH30* allele. The CTX-M is a family of ESBL enzymes originally described among human specimens in Germany and Argentina (Philippon et al. 1989; Paterson and Bonomo 2005). Chromosomal β-lactamase genes from *Kluyvera* spp., particularly *K. ascorbata* and *K. georgiana*, have been identified as potential sources of specific CTX-M genes (Rodríguez et al. 2004; Olson et al. 2005).

CTX-M-15, first identified in India in 1999 (Karim et al. 2001), is the most widely distributed CTX-M worldwide (Canton and Coque 2006). The CTX-M-15 enzyme is responsible for resistance to penicillins, cephalosporins (excluding the cephamycins), and monobactams (Rogers et al. 2011). Other CTX-M-type β-lactamases reported in association with the ST131 clone include CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-27, CTX-M-32, and CTX-M-61 (Clermont et al. 2009; Mora et al. 2010). CTX-M-15 is predominantly a plasmid-encoded gene among ST131 clones, although chromosomal location has also been reported (Price et al. 2013). ST131 clones carrying CTX-M-15 mobile elements may also carry resistance genes to different antibiotic classes, including carbapenems, fluoroquinolones, and aminoglycosides (Banerjee et al. 2013a). Fluoroquinolone resistance among ST131 R30 clones is not mediated by degrading enzymes, but rather, by amino acid substitutions at the quinolone resistance-determining regions of target GyrA and ParC proteins (Cagnacci et al. 2008; Johnson et al. 2013).

MLST typing involves the sequencing of seven housekeeping *E. coli* chromosomal genes. These genes, believed not to undergo positive selection, may undergo nucleotide mutations as a measure of time. Based on the type of sequences obtained for all seven alleles, the resulting pattern in the data base provides a unique sequence type number identified. *E. coli* strains containing identical sequence for each one of the seven alleles will have the same sequence type number. *E. coli* ST131 is a member of the ancestral phylogenetic clonal group B2, which diverged from clonal groups A and B1. Ancestral groups B2 and D are known to carry multiple virulence factors and pathogenicity islands that are critical during extra-intestinal infections. Most ST131 strains are serotype O25:H4, although other serotypes have also been described (Nicolas-Chanoine et al. 2014).

The global phylogeny of *E. coli* ST131, determined from whole-genome sequence data, from six distinct geographical locations across the world spanning 2000–2011, revealed a single lineage of *E. coli* ST131 distinct from other extra-intestinal *E. coli* strains within the B2 phylogroup. This lineage was characterized by fluoroquinolone resistance, and a distinct virulence factor and mobile genetic element profile (Petty et al. 2014). Phylogenomic analysis showed that fluoroquinolone resistance was initially limited to a single ST131 subclone, designated H30-R. This fluoroquinolone resistance clone was identified in North America in 1999, 4 years after fluoroquinolones were introduced in the United States for medical and veterinary use. Genomic analysis of publically available *E. coli* genomes made possible the evolutionary reconstruction of this subclone (Ben Zakour et al. 2016). It was demonstrated that fluoroquine resistance ST131 subclone acquired fitness and virulence genes prior to the acquisition of fluoroquinolone resistance. This order of events may explain, at least in part, the amazing ability of the strain to rapidly spread across the globe. Ninety-one percent of the CTX-M-15-producing isolates also belonged to a single clade nested within H30-R, designated H30-Rx, due to its more extensive antibiotic resistance. The CTX-M-15 mobile element in the H30-R subclone was inserted variably in plasmid and chromosomal locations within the H30-Rx genome. Screening of a large collection of recent *E. coli* clinical isolates confirmed the global clonal expansion of H30-Rx and revealed its disproportionate association with sepsis (relative risk 7.5; $p < 0.001$) (Johnson et al. 2016). Phylogenetic analysis indicated that the H30 ST131 lineage emerged 25 years ago and that sub-lineages (H30-R and H30-RX) continued to expand rapidly after that (Mathers et al. 2015).

In summary, molecular epidemiologic and whole-genome phylogenetic studies have elucidated the fine clonal structure of ST131, which comprises multiple ST131 subclones with distinctive resistance profiles, including the (nested) H30, H30-R, and H30-Rx subclones. The most prevalent ST131clone, H30, arose from a single common fluoroquinolone-susceptible ancestor containing allele 30 of *fimH* (type 1 fimbrial adhesin gene). An early H30 subclone member acquired fitness, and virulence genes and subsequently fluoroquinolone resistance, including genomic island GI-*pheV*, genomic island GI-*leuX*, *fimH30,* and IS*Ec55*. It is proposed that virulence gene acquisition was necessary prior to development of fluoroquinolone resistance for successful spread. This subclone launched the rapid expansion of the resulting fluoroquinolone-resistant subclone, H30-R. Subsequently, acquisition of the CTX-M-15 extended-spectrum beta-lactamase launched the rapid expansion of the CTX-M-15-containing subclone within H30-R, designated H30-Rx.

3.3 Relationship Between Antibiotic Resistance and Pathogenesis

Unlike other classical extra-intestinal *E. coli* (ExPEC) group B2 isolates, ST131 expresses ESBLs, predominantly CTX-M-15, and almost all are resistant to fluoroquinolones. Moreover, *E. coli* ST131 clones are pathogenic, due to the spectrum of

infections affecting community and hospital patients and due to the large number of virulence-associated genes they contain. The ST131 clones seem to contradict the theory that high levels of antimicrobial resistance are associated with limited fitness and consequently low virulence (Nicolas-Chanoine et al. 2014). Assessment of *E. coli* ST131 in Chicago area from 2007 to 2010 showed that ST131 H30 clones and H30-Rx subclones had increased number of antimicrobial resistance phenotype and a distinctive virulence profile than non-H30 ST131 clones. The *iha* adhesin, *sat* toxin, and *iutA* siderophore genes were frequently associated with ST131 H30 subclones (Banerjee et al. 2013a).

4 Antibiotic Resistance of *E. coli* Studies in the Americas

The Latin American region is recognized as facing a significant challenge with high levels of antimicrobial resistance among important Gram-negative organisms including *E. coli.* In recent years, extended-spectrum β-lactamases (ESBLs) have increased in type and frequency among *Enterobacteriaceae* and carbapenemases have emerged. In addition, multidrug resistance *E. coli* has spread across the American continent.

4.1 E. coli Antibiotic Resistance in North and Central America

4.1.1 Antibiotic Resistance of ExPEC

E. coli isolates resistant to all common antibiotics and also to carbapenems have become a challenge to clinicians confronted with higher morbidity and higher mortality among patient with *E. coli*-mediated infectious diseases. The increased level of resistance was nicely demonstrated when strains isolated from 1950 were compared with strains isolated in the 2000s to a total of 15 different antibiotics. In this study, it was also shown that multidrug resistance among *E. coli* strains increased from 7.2 % during the 1950s to 63.6 % by the 2000s (Tadesse et al. 2012).

In the US, the incidence of carbapenem-resistant *Enterobacteriaceae* was measured based on specimens collected from 2012 to 2013, in seven distinct US communities. It was found that the prevalence of carbapenem-resistant *Enterobacteriaceae* was ~4 positive subjects per 100,000 inhabitants. *Enterobacteriaceae* isolates, including *E. coli* and *K. pneumoniae*, were isolated predominantly from urine samples and subjects with indwelling devices and history of exposure to hospital environments (Guh et al. 2015). Multidrug-resistant *E. coli* previously limited to healthcare facilities, and predominantly intensive care units, have now expanded to the community where they have colonized humans, animals, and environmental sources. The multi-resistant phenotype is expressed among *E. coli* clinical isolates and includes commensal as well as extra-intestinal and intestinal pathogens (Mathers et al. 2015). *E. coli* clinical isolates from 24 US veteran medical centers reported

that 28 % of these *E. coli* isolates were members of the ST131 clone, followed by ST95 and ST12 (13 % each). ST131 accounted for ≥40 % of β-lactamase resistance, >50 % of trimethoprim-sulfamethoxazole resistance, and >70 % of ciprofloxacin and gentamicin resistance (Colpan et al. 2013). Surveillance studies on long-term care facilities by rectal swabs found that 25 % of subjects were positive for ST131 subclone H30 and all resistant to fluoroquinolones (Burgess et al. 2015).

The number of inpatient and outpatient infections by antibiotic-resistant organisms in the pediatric population is on the rise. In a survey of *Enterobacteriaceae* organisms from pediatric-associated extra-intestinal infections in the US, it was observed that the proportion of third-generation cephalosporins-resistant organisms increased from 1.39 % in 1999 to 3 % in 2011. Similarly, the proportion of ESBL increased from 0.28 % to 0.92 % in 2011. The majority of these organisms were *E. coli* isolated from blood, urine, respiratory, or wound cultures (Logan et al. 2014). Reports of antibiotic-resistant *E. coli* associated with neonatal sepsis indicate that they may be up to 75 % resistant to ampicillin and 17 % resistant to gentamicin (Shakir et al. 2014). Considering that the traditional antibiotic regimen for neonatal sepsis remains ampicillin and gentamicin and that an increased proportion of extraintestinal *E. coli* infections are highly resistant to these antibiotics, strong consideration for alternative regimens should be sought. A recent case report of an ampicillin- and gentamicin-resistant *E. coli* confirms that the current empiric antibiotic coverage may lead to poor outcomes, especially in cases of *E. coli* meningitis where the severity of the infection is very high (Iqbal et al. 2016).

A study evaluating the proportion of multidrug-resistant *E. coli* colonization in mothers and their babies found that a total of 20 % of mothers and 19 % of babies were positive for ciprofloxacin-resistant *E. coli* strains. ST131 and ST405 were the predominant *E. coli* clones. Length of hospital stay after birth ($p = 0.002$) and maternal colonization ($p = 0.0001$) were associated with subsequent childhood carriage of resistant *E. coli* (Gurnee et al. 2015). The prevalence of resistance varies according with geographic region. *E. coli* clinical isolates from a tertiary pediatric-care center in Houston, TX, identified 6.5 % ESBL positive isolates. The genes encoding ESBL were predominantly CTX-M, and in 80 % of the cases, they were CTX-M-15, followed by CTX-M-14 (17 %) and CTX-M-27 (10.2 %) (Chandramohan and Revell 2012). *E. coli* clinical isolates from cases of neonatal sepsis in Washington State were evaluated for resistance and 78 % of them were ampicillin-resistant. ESBL and fluoroquinolone resistance was identified among ST131 *E. coli* clones and no aminoglycoside resistance was detected in this study (Weissman et al. 2015).

E. coli is a common gut colonizer of large and small mammalian species and, as expected, bovine, canines, among other animals, are colonized with *E. coli*. Direct contact with animals is therefore a significant risk factor for colonization by multidrug-resistant *E. coli* strains. Antimicrobial susceptibility testing performed on 3373 bovine *E. coli* isolates from clinical samples in United States showed 2.7 % resistance to enrofloxacin, a fluoroquinolone antibiotic approved for animal use and 91.3 % resistance to oxytetracycline. Although, decreasing resistance trends were noticed with tetracyclines, aminoglycosides, and trimethoprim/sulfamethoxazole, the increased level of resistance to fluoroquinolones was concerning (Cummings et al. 2014).

Reports from Mexico indicate that *E. coli* is a common cause of intestinal infections. In one study, a total of 4735 *E. coli* isolates from urine samples were tested for antimicrobial resistance. A 10.2 % of all the strains were ESBL producers and CTX-M_15 was carried by 94.4 % of the strains. Other ESBL genes included SHV-2a in 3 % and TLA-1 in 1 % of the isolates. The majority of the isolates (94.6 %) were also positive for plasmid-mediated quinolone resistance genes (*aac* (6′)-*lb-cr* [92.1 %] and *qepA1* [7 %] and for quinolone resistant determinant [3.5 %]) (Reyna-Flores et al. 2013). In a more recent study also evaluating *E. coli* isolates from patient with UTI, *E. coli* ST131 serotype O25 accounted for 21.5 % of all isolates (Miranda-Estrada et al. 2016).

4.1.2 Antibiotic Resistance of Intestinal *E. coli*

E. coli is a common cause of intestinal infections in Mexico and Central America and to a lesser extent in the US. Literature on multidrug resistance among ExPEC pathogens is extensive, and it's based on the analysis of *E. coli* strains isolated from extra-intestinal sites. The reservoir of these multi-resistant *E. coli*, however, is the host gut. Intestinal *E. coli* pathotypes in the US showed resistance to antibiotics. In a recent study conducted in the middle Tennessee, US, the proportion of ampicillin-resistant *E. coli* intestinal pathotypes was 41.6 % and 2 % of them were multidrug-resistant (Foster et al. 2015).

Evaluation of intestinal *E. coli* pathogens obtained from Costa Rican children with diarrhea revealed that 40 % of the isolates were resistant to ampicillin, and in less proportion to ticarcillin (45), and cephalosporins 1–3 % (Pérez et al. 2010). An important public health concern in Mexico is not only the consumption of food contaminated with *E. coli,* but also the level of antimicrobial resistance of the contaminating *E. coli* strains. The STEC O157 and non-O157 strains recovered from farm animals exhibited resistance to aminoglycosides, tetracyclines, cephalosporins, and penicillin (Amézquita-López et al. 2016). *E. coli* isolated from bovine carcasses identified genes encoding ESBL genes bla_{CMY}, bla_{TEM}, as well as quinolone resistance genes *qnrB* and *qnrS* (Aguilar-Montes de Oca et al. 2015). Whether these strains colonize the human gut or horizontally transfer antimicrobial resistance genes to human gut colonizers is a question that remains to be answered.

4.2 Antibiotic Resistance of E. coli in South America

The Americas have a regional surveillance network, the Latin American Network for Antimicrobial Resistance Surveillance, which coordinates surveillance in 21 countries (World Health Organization 2014). However, few countries have a national plan with policies and strategies for controlling antimicrobial resistance (World Health Organization 2015). The Regional Technical Advisory Group on Antimicrobial Resistance and Infection Prevention and Control suggested that a framework be developed to help countries construct national plans (Pan American Health Organization 2013).

4.2.1 Antibiotic Resistance of ExPEC

The Latin American Antimicrobial Resistance Surveillance Network (ReLAVRA), led by the Pan American Health Organization (PAHO), collects aggregated data provided by national reference laboratories. A recent ReLAVRA study from 14 countries indicates that the overall reported range of *E. coli* resistance to third-generation cephalosporins is between 0–48 % and to fluoroquinolones is 8–58 % (World Health Organization 2014).

A surveillance study of antimicrobial susceptibility of Gram-negative isolates was conducted in 12 countries in Latin America between 2004 and 2010 (Fernandez-Canigia and Dowzicky 2012). The Gram-negative isolates submitted were considered "clinically significant" using local criteria; the sources included blood, respiratory tract, urine (<25 % of all isolates), skin, wound, and fluids. A total of 3581 *E. coli* isolated were analyzed; 24.3 % were identified as ESBL producers (870/3581). The ESBL rates show important variations by country: 13.1 % in Argentina (101/769), 17.4 % in Brazil (43/247), 34.7 % in Chile (94/271), 11.2 % in Colombia, (58/519), 30.8 % in Guatemala (81/263), 40.3 % in Honduras (31/77), 38.1 % in Mexico (398/1044), 16 % in Panama (16/100), and 14.7 % in Venezuela (32/218). Among non-ESBL *E. coli* strains, resistance to amikacin was 1.7 %, ampicillin 69.9 %, cefepime 3.6 %, ceftriaxone 15.6 %, levofloxacin 36.2 %, meropenen 1.0 %, and piperacillin/tazobactam (pip/taz) 6.0 %; and among ESBL *E. coli* strains, amikacin 5.3 %, levofloxacin 85.1 %, meropenen 2.3 %, and pip/taz 10.0 % (Fernandez-Canigia and Dowzicky 2012). In a similar study conducted in 11 Latin American countries during 2008–2009, among 1366 *E. coli* strains isolated from intra-abdominal infections, the prevalence of ESBL was 23.6 % (Hawser et al. 2012).

Intensive care unit (ICU) facilities are typically associated with high rates of resistant organisms. Among 532 *E. coli* strains isolated from 65 ICUs in Latin America between 2004 and 2009, the rate of ESBL was 25.9 % (Bertrand and Dowzicky 2012). Resistance to amikacin was 6.0 %, ampicillin 75.6 %, cefepime 20.9 %, ceftriaxone 35.2 %, levofloxacin 46.1 %, meropenem 2.9 %, and piperacillin/tazobactam 15.8 % (Bertrand and Dowzicky 2012). A descriptive study was conducted in ICUs of 23 hospitals in Colombia over a 4-year period. *E. coli* was the most frequently isolated organism. Multidrug-resistant *E. coli* (ESBLs and resistant to ciprofloxacin) increased from 62 % in 2009 to 79 % in 2012 (Hernández-Gómez et al. 2014). Another study in Colombia found that the main risk factors for infection or colonization by ESBL *E. coli* were chronic renal failure, urologic surgery, antibiotic use in the previous 3 months, nosocomial origin of infection, and previous hospitalization (Jiménez et al. 2014).

E. coli is an important cause of sepsis and meningitis in neonates. A study in Argentina found 14.3 % prevalence of vaginal *E. coli* colonization during pregnancy. The resistance rate to ampicillin was 49 % and to gentamicin 11 %; five percent of pregnant women were colonized with *E. coli* ESBL-producer strains (Villar et al. 2013). Thus, screening strategies designed to monitor for ESBL-producing *E. coli* could be useful in endemic areas to prevent perinatal transmission and the introduction of multi-resistant strains to the maternity ward.

The antibiotic resistance of *E. coli* strains isolated from urinary tract infection (UTI) is high to commonly used antibiotics. A recent surveillance study conducted at the Emergency Room of a Public Hospital in Lima, Peru, during 2012–2013 found high antimicrobial resistance rates on 357 *E. coli* strains isolated from urinary tract infections (61 from pediatric patients and 296 from adults) (Curi et al. 2014). Resistance to ampicillin was 84 %, ciprofloxacin 59 %, and ceftriaxone 44 %. There was less resistance to nitrofurantoin (3 %) and amikacin (5 %). Resistance to ciprofloxacin was significantly lower among pediatric patients than adults (34 % vs. 64 %, $p < 0.001$) (Curi et al. 2014). In Colombia, among 104 *E. coli* strains isolated from pediatric patients with UTI for the period 2010–2011, resistance to ciprofloxacin was 7 %, TMP-SXT 43 %, ampicillin-sulbactam 48 %, and ampicillin 63 % (Vélez Echeverri et al. 2014). Among 739 *E. coli* strains isolated from UTI in Santa Cruz do Sul in Brazil, between 2004–2007, the resistance rate to ampicillin was around 50 % and to TMP-SXT around 45 %; the *sul2* gene was present in 67 % of resistant strains. Resistance rates were higher in hospitalized patients in comparison with ambulatory patients (Teichmann et al. 2014).

4.2.2 Antibiotic Resistance of Intestinal *E. coli*

Bartoloni and colleagues, in a 20-year surveillance study in a resource-limited setting in Bolivia, found an important increase in the rate of fluoroquinolones and ESBL *E. coli* resistance (Bartoloni et al. 2013). In the last studied period (2011), 482 rectal swabs from healthy children <6 years of age, from three urban areas of the Bolivian Chaco, were studied; high rates of resistance to nalidixic acid (76 %), ciprofloxacin (44 %), and expanded-spectrum cephalosporins (12.4 %) were found. Compared with the previous periods, CTX-M-producing *E. coli* had a 120-fold increase since early 2000s. There was a significant change of dominant CTX-M groups (CTX-M-1 and CTX-M-9 groups vs. CTX-M-2 group). Most CTX-M producers were not susceptible to quinolones (91 %), and 55 % carried plasmid-mediated quinolone resistance genes (Bartoloni et al. 2013).

A 4-year study in five hospitals in Bolivia analyzed around 4000 stool samples from children hospitalized with diarrhea and around 1000 control samples of children without diarrhea (Gonzales et al. 2013). The prevalence of diarrheagenic *E. coli* (DEC) (including enteroaggregative [EAEC], enteropathogenic [EPEC], enterotoxigenic [ETEC], enteroinvasive [EIEC], and enterohemorrhagic *E. coli* [EHEC]) was higher among diarrhea than control cases (21.6 % vs. 17.6 %, $p < 0.002$). The antibiotic resistance rate was high for ampicillin (94 %), TMP-SMX (75 %), tetracycline (55 %), and amoxicillin-sulbactam (41 %). Low resistance rates were observed for chloramphenicol, nalidixic acid, cefotaxime, and ciprofloxacin (Fig. 13.1). Resistance to TM-SMX was higher among DEC-diarrhea than DEC control (75 % vs. 51 % $p = 0.001$). Multi-drug resistance was present in 62.2 % of strains (Gonzales et al. 2013).

Mosquito and colleagues reported the antimicrobial resistance mechanism of DEC isolated from Peruvian children younger than 12 months of age from peri-urban areas of Lima during 2006–2007 (Mosquito et al. 2012). A total of 369 *E. coli*

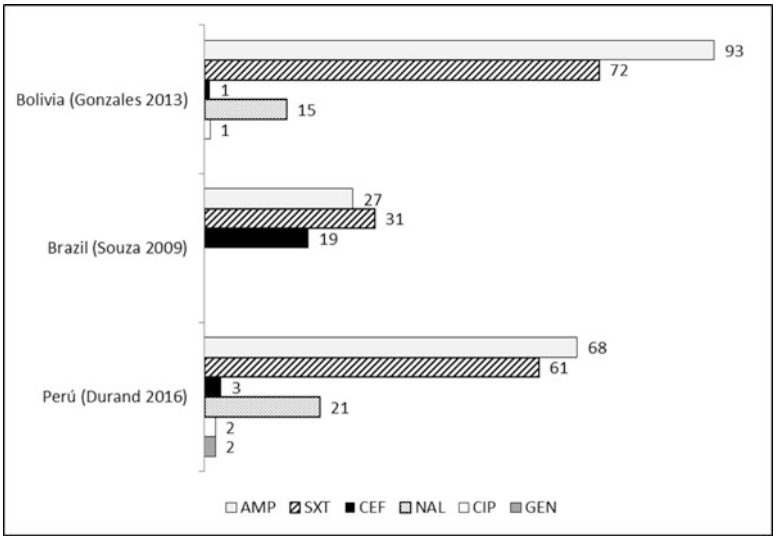

Fig. 13.1 Antimicrobial resistance of diarrheagenic *E. coli* strains from South America. From studies published in PubMed in the last 10 years. Bolivia ($n=986$ strains), Brazil ($n=52$ strains), and Peru ($n=776$ strains). *AMP* ampicillin, *SXT* trimethoprim/sulfamethoxazole, *CEF* ceftriaxone, *NAL* nalidixic acid, *CIP* ciprofloxacin, *GEN* gentamicin

strains were analyzed, including 74 commensal *E. coli*, 94 DEC control (isolated from children without diarrhea), and 201 DEC diarrhea strains. The DEC group included EPEC, ETEC, EAEC, and diffuse adherent *E. coli* (DAEC) pathogroups. In general, *E. coli* strains were commonly resistant to ampicillin (80 %), SXT (71 %), tetracycline (56 %), chloramphenicol (21 %), and nalidixic acid (32 %). Quinolone (nalidixic acid and ciprofloxacin) resistance was significantly higher in commensal strains than in DEC strains ($p<0.05$). Multi-resistance was found in 76 % of EAEC and 90 % of DAEC. A total of 19 mechanisms of resistance and two integrases were searched in this study. The most frequent mechanisms of antibiotic resistance in DEC strains included: for β-lactams, bla_{TEM} (31 %; 37/118); for SXT, *sul2* (48 %; 49/103); for tetracycline, *tetA* (27 %; 23/84); and for chloramphenicol, *cat* (80 %; 28/35). The genes *sul1* and *dfrA1*, related to SXT resistance, were more frequent in the DEC diarrhea group than in DEC control (41 % and 28 %, respectively, $p<0.05$). The most common resistance genes detected among commensal strains were *cat* (70 %), *sul2* (56 %), *floR* (30 %), and bla_{TEM} (28 %). The most frequent mechanisms of antibiotic resistance gene transmission were directly associated with mobile DNA elements. This mechanism of transmission is known to rapidly spread within the host and between hosts and it explains, at least in part, the high prevalence of antibiotic-resistant *E. coli* in the study population.

A recent study reported the antimicrobial resistance rates of 776 diarrheagenic *E. coli* strains isolated from children < 24 months of age with diarrhea (533 strains) and from healthy controls (243 strains) from peri-urban Lima during 2008–2011 (Durand et al. 2016). The study sample included 346 EPEC, 253 EAEC, and 177 ETEC strains. Resistance to ampicillin was 68 %, TMP-SMX 61 %, and tetracycline

49 %. However, resistance to ciprofloxacin and ceftriaxone was less than 3 %. Of interest, resistance to nalidixic acid was 21 % and azithromycin 8 % (Fig. 13.1). In regard to quinolone resistance genes (*qnr*), the *qnrB* genes are widely distributed in South American countries; including children in urban settings in Peru and Bolivia and humans in remote Peruvian Amazonian communities (Pallecchi et al. 2009, 2011). Of interest, the *qnrB* genes carriage rate in Ecuador was higher in healthy humans from rural communities than urban areas (Armas-Freire et al. 2015). Among 139 EAEC strains isolated from infants in peri-urban communities of Lima, Peru, 32 % were resistant to nalidixic acid and 2 % resistant to ciprofloxacin. On these strains, several plasmid-mediated quinolone resistance (PMQR) genes were searched yet, only *qnrB*19 was detected in seven isolates (5 %) (Riveros et al. 2012).

These findings emphasize the role of gut microbiota as a reservoir of resistance genes potentially transferable to enteric pathogens, especially to those that may establish successful long-term gut colonization in the human host. Additionally, these studies highlight the importance of persistent surveillance studies in Latin American countries, where the most commonly used antibiotics in children and adults are available without prescription (Wirtz et al. 2010).

4.3 Summary and Implications for Management and Stewardship Programs

Antimicrobial resistance, including that of *E. coli*, is associated with increasing morbidity and mortality among adult and pediatric patients at in-patient and outpatient facilities worldwide. In addition, treating infections caused by multiresistant organism has dramatically increased the health care cost as well as the cost to society due to lack of productivity. Spread of *E. coli* antimicrobial resistance underscores the importance of antimicrobial stewardship programs, since inappropriate use of antibiotics directly influences the development of antimicrobial resistance.

Guidelines on proper antibiotic use in the era of multidrug resistance are critical, not only to optimize clinical outcomes, but also to lower the proportion of antibiotic resistance. The Infectious Diseases Society of America recommends the establishment of antimicrobial stewardship programs at every medical center where primary objectives are to improve clinical outcomes, minimize antibiotic resistance, and decrease healthcare costs (Dellit et al. 2007). Antimicrobial stewardship programs require a team of individuals with expertise in infectious diseases, antibiotics use, microbiology, and epidemiology and it is generally conformed by an infectious diseases physician and a clinical pharmacologist. Key antimicrobial stewardship team activities and infection control activities are necessary for the success of the program. The antimicrobial stewardship team and the local clinical microbiology laboratory should create, and continuously update, an antibiogram database to determine the level of resistance among bacterial agents, including *E. coli*. This data may help guide empiric management of *E. coli* extra-intestinal infections. The antimicrobial

stewardship team, in coordination with the medical center administration and treating physicians, should establish parameters, guides, and algorithms to control the use of antibiotics in real-time and to provide feedback to treating clinicians on the regular basis (Cosgrove et al. 2014). The program should also establish well-defined antibiotic restriction and antibiotic preauthorization for certain antibiotics (Barlam et al. 2016). In the case of *E. coli*, antibiotics that may be restricted include carbapemens, tigacycline, amikacin, certoroline, ceftazidime-avibactam, among others. The goal of this recommendation is to prevent inappropriate use of antibiotics, decrease the dose of antibiotics when appropriate, and to limit the time antibiotics are used for certain infections.

Information on the local *E. coli* susceptibility may also contribute to the establishment of guidelines for empiric use of antibiotics for specific infectious conditions or specific populations (Barlam et al. 2016). The antimicrobial stewardship program may help establish guidelines or algorithms for use of empiric antibiotics for neonatal sepsis and determine, based on local *E. coli* susceptibility patterns, whether ampicillin-gentamicin combination, ampicillin-cefotaxime, or alternative options should be considered. Similarly, antibiotic-recommended guidelines may be established for community-acquired or ventilator-acquired pneumonia, sepsis, UTI, and meningitis, in which *E. coli* is the suspected pathogen. Although third-generation cephalosporin resistance and ESBL resistance among *E. coli* clinical isolates are well-known, the level of antibiotic resistance varies from place to place and only antibiogram data from *E. coli* clinical isolates plus survey from healthy carries on antibiotic susceptibility may provide key information to base further antibiotic recommendations to adult and pediatric patients and at any geographic location.

References

Aguilar-Montes de Oca S, Talavera-Rojas M, Soriano-Vargas E, Barba-León J, Vazquez-Navarrete J (2015) Determination of extended spectrum β-lactamases/AmpC β-lactamases and plasmid-mediated quinolone resistance in *Escherichia coli* isolates obtained from bovine carcasses in Mexico. Trop Anim Health Prod 47:975–981

Amézquita-López BA, Quiñones B, Soto-Beltrán M, Lee BG, Yambao JC, Lugo-Melchor OY, Chaidez C (2016) Antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* O157 and Non-O157 recovered from domestic farm animals in rural communities in Northwestern Mexico. Antimicrob Resist Infect Control 5:1

Amos GCA, Hawkey PM, Gaze WH, Wellington EM (2014) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. J Antimicrob Chemother 69:1785–1791

Armas-Freire PI, Trueba G, Proano-Bolanos C, Levy K, Zhang L, Marrs CF, Cevallos W, Eisenberg JNS (2015) Unexpected distribution of the fluoroquinolone-resistance gene qnrB in *Escherichia coli* isolates from different human and poultry origins in Ecuador. Int Microbiol 18:85–90

Banerjee R, Johnson JR (2014) A new clone sweeps clean: the enigmatic emergence of *Escherichia coli* sequence type 131. Antimicrob Agents Chemother 58:4997–5004

Banerjee R, Robicsek A, Kuskowski MA, Porter S, Johnston BD, Sokurenko E, Tchesnokova V, Price LB, Johnson JR (2013a) Molecular epidemiology of *Escherichia coli* sequence type 131 and Its H30 and. Antimicrob Agents Chemother 57:6385–6388

Banerjee R, Strahilevitz J, Johnson JR, Nagwekar PP, Schora DM, Shevrin I, Du H, Peterson LR, Robicsek A (2013b) Predictors and molecular epidemiology of community-onset extended-spectrum beta-lactamase-producing *Escherichia coli* infection in a Midwestern community. Infect Control Hosp Epidemiol 34:947–953

Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Matter LB, Pinheiro SRS, Vaz TMI, Nolan LK, Logue CM, de Brito BG, Horn F (2015) Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathog Dis 12:74–83

Barlam TF, Cosgrove SE, Abbo LM, MacDougall C, Schuetz AN, Septimus EJ, Srinivasan A, Dellit TH, Falck-Ytter YT, Fishman NO, Hamilton CW, Jenkins TC, Lipsett PA, Malani PN, May LS, Moran GJ, Neuhauser MM, Newland JG, Ohl CA, Samore MH, Seo SK, Trivedi KK (2016) Implementing an antibiotic stewardship program: guidelines by the infectious diseases society of America and the society for healthcare epidemiology of America. Clin Infect Dis 62:e51–e77

Bartoloni A, Pallecchi L, Riccobono E, Mantella A, Magnelli D, Di Maggio T, Villagran AL, Lara Y, Saavedra C, Strohmeyer M, Bartalesi F, Trigoso C, Rossolini GM (2013) Relentless increase of resistance to fluoroquinolones and expanded-spectrum cephalosporins in *Escherichia coli*: 20 years of surveillance in resource-limited settings from Latin America. Clin Microbiol Infect 19:356–361

Ben Zakour NL, Alsheikh-Hussain AS, Ashcroft MM, Khanh Nhu NT, Roberts LW, Stanton-Cook M, Schembri MA, Beatson SA (2016) Sequential acquisition of virulence and fluoroquinolone resistance has shaped the evolution of *Escherichia coli* ST131. MBio 7(3):e00347-16

Bertrand X, Dowzicky MJ (2012) Antimicrobial susceptibility among gram-negative isolates collected from intensive care units in North America, Europe, the Asia-Pacific Rim, Latin America, the Middle East, and Africa between 2004 and 2009 as part of the Tigecycline Evaluation and Surveillance Trial. Clin Ther 34:124–137

Beyer A, Baumann S, Scherz G, Stahl J, von Bergen M, Friese A, Roesler U, Kietzmann M, Honscha W (2015) Effects of ceftiofur treatment on the susceptibility of commensal porcine *E. coli*—comparison between treated and untreated animals housed in the same stable. BMC Vet Res 11:265

Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51

Blanco M, Blanco JE, Blanco J, Gonzalez EA, Mora A, Prado C, Fernandez L, Rio M, Ramos J, Alonso MP (1996) Prevalence and characteristics of *Escherichia coli* serotype O157:H7 and other verotoxin-producing *E. coli* in healthy cattle. Epidemiol Infect 117:251–257

Buchanan R, Stoesser N, Crook D, Bowler IC. Multidrug-resistant *Escherichia coli* soft tissue infection investigated with bacterial whole genome sequencing. BMJ Case Rep. 2014 Oct 19; 2014. pii: bcr2014207200. doi:10.1136/bcr-2014-207200

Burgess MJ, Johnson JR, Porter SB, Johnston B, Clabots C, Lahr BD, Uhl JR, Banerjee R (2015) Long-term care facilities are reservoirs for antimicrobial-resistant sequence type 131 *Escherichia coli*. Open Forum Infect Dis 2(1):ofv011

Bush K (2013a) Proliferation and significance of clinically relevant beta-lactamases. Ann N Y Acad Sci 1277:84–90

Bush K (2013b) The ABCD's of beta-lactamase nomenclature. J Infect Chemother 19:549–559

Bush K, Jacoby GA (2010) Updated functional classification of beta-lactamases. Antimicrob Agents Chemother 54:969–976

Cagnacci S, Gualco L, Debbia E, Schito GC, Marchese A (2008) European emergence of ciprofloxacin-resistant *Escherichia coli* clonal groups O25:H4-ST 131 and O15:K52:H1 causing community-acquired uncomplicated cystitis. J Clin Microbiol 46:2605–2612

Can F, Azap OK, Seref C, Ispir P, Arslan H, Ergonul O (2015) Emerging *Escherichia coli* O25b/ST131 clone predicts treatment failure in urinary tract infections. Clin Infect Dis 60:523–527

Canton R, Coque TM (2006) The CTX-M beta-lactamase pandemic. Curr Opin Microbiol 9:466–475

Castanon JIR (2007) History of the use of antibiotic as growth promoters in European poultry feeds. Poult Sci 86:2466–2471

Chandramohan L, Revell PA (2012) Prevalence and molecular characterization of extended-spectrum-β-lactamase-producing *Enterobacteriaceae* in a pediatric patient population. Antimicrob Agents Chemother 56:4765–4770

Cho SY, Kang C-I, Cha MK, Wi YM, Ha YE, Chung DR, Lee NY, Peck KR, Song J-H (2015) Clinical features and treatment outcomes of bloodstream infections caused by extended-spectrum beta-lactamase-producing *Escherichia coli* sequence type 131. Microb Drug Resist 21:463–469

Clermont O, Dhanji H, Upton M, Gibreel T, Fox A, Boyd D, Mulvey MR, Nordmann P, Ruppé E, Sarthou JL, Frank T, Vimont S, Arlet G, Branger C, Woodford N, Denamur E (2009) Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. J Antimicrob Chemother 64:274–277

Colpan A, Johnston B, Porter S, Clabots C, Anway R, Thao L, Kuskowski MA, Tchesnokova V, Sokurenko EV, Johnson JR, VICTORY (Veterans Influence of Clonal Types on Resistance: Year 2011) Investigators (2013) *Escherichia coli* sequence type 131 (ST131) subclone H30 as an emergent multidrug-resistant pathogen among US veterans. Clin Infect Dis 57:1256–1265

Cosgrove SE, Hermsen ED, Rybak MJ, File TM, Parker SK, Barlam TF, Society for Healthcare Epidemiology of America, Infectious Diseases Society of America, Making-A-Difference in Infectious Diseases, National Foundation of Infectious Diseases, Pediatric Infectious Diseases Society, Society of Infectious Disease Pharmacists (2014) Guidance for the knowledge and skills required for antimicrobial stewardship leaders. Infect Control Hosp Epidemiol 35:1444–1451

Cummings KJ, Aprea VA, Altier C (2014) Antimicrobial resistance trends among *Escherichia coli* isolates obtained from dairy cattle in the northeastern United States, 2004–2011. Foodborne Pathog Dis 11:61–67

Curello J, MacDougall C (2014) Beyond susceptible and resistant, part II: treatment of infections due to gram-negative organisms producing extended-spectrum beta-lactamases. J Pediatr Pharmacol Ther 19:156–164

Curi B, Torres C, Yabar M (2014) Prevalencia de *E. coli* diarreogénicas en urocultivos de pacientes con diagnóstico de Infección del tracto urinario por *E. coli* en el Hospital Nacional Cayetano Heredia. Tesis para optar el grado de Bachiller en Medicina, Universidad Peruana Cayetano Heredia, Lima, Peru

Dellit TH, Owens RC, McGowan JE, Gerding DN, Weinstein RA, Burke JP, Huskins WC, Paterson DL, Fishman NO, Carpenter CF, Brennan PJ, Billeter M, Hooton TM, Infectious Diseases Society of America, Society for Healthcare Epidemiology of America (2007) Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship. Clin Infect Dis 44:159–177

Durand D, Contreras CA, Mosquito S, Ruíz J, Cleary TG, Ochoa TJ. pic gene of enteroaggregative *Escherichia coli* and its association with diarrhea in Peruvian children. Pathog Dis. 2016 Aug;74(6). pii: ftw054. doi:10.1093/femspd/ftw054. Epub 2016 Jun 14

Fadlelmula A, Al-Hamam NA, Al-Dughaym AM (2016) A potential camel reservoir for extended-spectrum β-lactamase-producing *Escherichia coli* causing human infection in Saudi Arabia. Trop Anim Health Prod 48:427–433

Fernandez-Canigia L, Dowzicky MJ (2012) Susceptibility of important Gram-negative pathogens to tigecycline and other antibiotics in Latin America between 2004 and 2010. Ann Clin Microbiol Antimicrob 11:29

Foster MA, Iqbal J, Zhang C, McHenry R, Cleveland BE, Romero-Herazo Y, Fonnesbeck C, Payne DC, Chappell JD, Halasa N, Gómez-Duarte OG (2015) Enteropathogenic and enteroaggregative *E. coli* in stools of children with acute gastroenteritis in Davidson County, Tennessee. Diagn Microbiol Infect Dis 83:319–324

Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1:191–203

Giuffrè M, Cipolla D, Bonura C, Geraci DM, Aleo A, Di Noto S, Nociforo F, Corsello G, Mammina C (2013) Outbreak of colonization by extended-spectrum β-lactamase-producing *Escherichia coli* sequence type 131 in a neonatal intensive care unit, Italy. Antimicrob Resist Infect Control 2:8

Gonzales L, Joffre E, Rivera R, Sjöling Å, Svennerholm A-M, Iñiguez V (2013) Prevalence, seasonality and severity of disease caused by pathogenic *Escherichia coli* in children with diarrhoea in Bolivia. J Med Microbiol 62:1697–1706

Guh AY, Bulens SN, Mu Y, Jacob JT, Reno J, Scott J, Wilson LE, Vaeth E, Lynfield R, Shaw KM, Vagnone PMS, Bamberg WM, Janelle SJ, Dumyati G, Concannon C, Beldavs Z, Cunningham M, Cassidy PM, Phipps EC, Kenslow N, Travis T, Lonsway D, Rasheed JK, Limbago BM, Kallen AJ (2015) Epidemiology of carbapenem-resistant *Enterobacteriaceae* in 7 US Communities, 2012–2013. JAMA 314:1479–1487

Gurnee EA, Ndao IM, Johnson JR, Johnston BD, Gonzalez MD, Burnham C-AD, Hall-Moore CM, McGhee JE, Mellmann A, Warner BB, Tarr PI (2015) Gut colonization of healthy children and their mothers with pathogenic ciprofloxacin-resistant *Escherichia coli*. J Infect Dis 212:1862–1868

Hawser SP, Bouchillon SK, Hoban DJ, Badal RE, Hackel MA, Lascols CA, Villegas MV, Rossi F (2012) Low frequency of ertapenem-resistant intra-abdominal isolates of *Escherichia coli* from Latin America: susceptibility, ESBL-occurrence, and molecular characterization (SMART 2008-2009). J Chemother 24:6–11

Hernández-Gómez C, Blanco VM, Motoa G, Correa A, Vallejo M, Villegas MV, Grupo de Resistencia Bacteriana Nosocomial en Colombia (2014) Evolution of antimicrobial resistance in Gram negative bacilli from intensive care units in Colombia. Biomedica 34(Suppl 1):91–100

Ho P-L, Lo W-U, Lai EL, Law PY, Leung SM, Wang Y, Chow K-H (2015) Clonal diversity of CTX-M-producing, multidrug-resistant *Escherichia coli* from rodents. J Med Microbiol 64:185–190

Iqbal J, Dufendach KR, Wellons JC, Kuba MG, Nickols HH, Gómez-Duarte OG, Wynn JL (2016) Lethal neonatal meningoencephalitis caused by multi-drug resistant, highly virulent *Escherichia coli*. Infect Dis 48:461–466

Jiménez A, Alvarado A, Gómez F, Carrero G, Fajardo C (2014) Risk factors associated with the isolation of extended spectrum betalactamases producing *Escherichia coli* or *Klebsiella* pneumoniae in a tertiary care hospital in Colombia. Biomedica 34(Suppl 1):16–22

Johnson JR, Drawz SM, Porter S, Kuskowski MA (2013) Susceptibility to alternative oral antimicrobial agents in relation to sequence type ST131 status and Coresistance phenotype among recent *Escherichia coli* isolates from U.S. veterans. Antimicrob Agents Chemother 57:4856–4860

Johnson JR, Thuras P, Johnston BD, Weissman SJ, Limaye AP, Riddell K, Scholes D, Tchesnokova V, Sokurenko E (2016) The pandemic H30 subclone of *Escherichia coli* sequence type 131 is associated with persistent infections and adverse outcomes independent from its multidrug resistance and associations with compromised hosts. Clin Infect Dis 62:1529–1536

Karim A, Poirel L, Nagarajan S, Nordmann P (2001) Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. FEMS Microbiol Lett 201:237–241

Logan LK, Braykov NP, Weinstein RA, Laxminarayan R, Epicenters Prevention Program CDC (2014) Extended-spectrum β-lactamase-producing and third-generation cephalosporin-resistant *Enterobacteriaceae* in children: trends in the United States, 1999–2011. J Pediatr Infect Dis Soc 3:320–328

Louie M, Cockerill FR 3rd (2001) Susceptibility testing. Phenotypic and genotypic tests for bacteria and mycobacteria. Infect Dis Clin North Am 15:1205–1226

Mathers AJ, Peirano G, Pitout JDD (2015) *Escherichia coli* ST131: the quintessential example of an international multiresistant high-risk clone. Adv Appl Microbiol 90:109–154

Millar MR, Seale J, Turton J, Wilks M, Costeloe K, Woodford N, Juszczak E, Whiley A, Panton N, Wareham DW (2016) ESBL-producing *Enterobacteriaceae* in 24 neonatal units and associated networks in the south of England: no clustering of ESBL-producing *Escherichia coli* in units or networks. J Antimicrob Chemother 71:1174–1177

Miranda-Estrada LI, Ruíz-Rosas M, Molina-López J, Parra-Rojas I, González-Villalobos E, Castro-Alarcón N. [Relationship between virulence factors, resistance to antibiotics and phylogenetic groups of uropathogenic *Escherichia coli* in two locations in Mexico]. Enferm Infecc Microbiol Clin. 2016 Apr 2. pii: S0213-005X(16)30006-4. doi:10.1016/j.eimc.2016.02.021. [Epub ahead of print]

Mora A, Herrera A, Mamani R, López C, Alonso MP, Blanco JE, Blanco M, Dahbi G, García-Garrote F, Pita JM, Coira A, Bernárdez MI, Blanco J (2010) Recent emergence of clonal group O25b:K1:H4-B2-ST131 *ibeA* strains among *Escherichia coli* poultry isolates, including CTX-M-9-producing strains, and comparison with clinical human isolates. Appl Environ Microbiol 76:6991–6997

Mosquito S, Ruiz J, Bauer JL, Ochoa TJ (2011) Molecular mechanisms of antibiotic resistance in *Escherichia coli*-associated diarrhea. Rev Peru Med Exp Salud Publica 28:648–656

Mosquito S, Ruiz J, Pons MJ, Durand D, Barletta F, Ochoa TJ (2012) Molecular mechanisms of antibiotic resistance in diarrhoeagenic *Escherichia coli* isolated from children. Int J Antimicrob Agents 40:544–548

Nicolas-Chanoine M-H, Bertrand X, Madec J-Y (2014) *Escherichia coli* ST131, an intriguing clonal group. Clin Microbiol Rev 27:543–574

Olson AB, Silverman M, Boyd DA, McGeer A, Willey BM, Pong-Porter V, Daneman N, Mulvey MR (2005) Identification of a progenitor of the CTX-M-9 group of extended-spectrum beta-lactamases from Kluyvera georgiana isolated in Guyana. Antimicrob Agents Chemother 49:2112–2115

Pallecchi L, Riccobono E, Mantella A, Bartalesi F, Sennati S, Gamboa H, Gotuzzo E, Bartoloni A, Rossolini GM (2009) High prevalence of qnr genes in commensal enterobacteria from healthy children in Peru and Bolivia. Antimicrob Agents Chemother 53:2632–2635

Pallecchi L, Riccobono E, Mantella A, Fernandez C, Bartalesi F, Rodriguez H, Gotuzzo E, Bartoloni A, Rossolini GM (2011) Small *qnrB*-harbouring ColE-like plasmids widespread in commensal enterobacteria from a remote Amazonas population not exposed to antibiotics. J Antimicrob Chemother 66:1176–1178

Pan American Health Organization (2013) PAHO Technical Advisory Group on antimicrobial resistance and infection prevention and control: biennial meeting final report. Washington, DC

Paterson DL, Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18:657–686

Pérez C, Gómez-Duarte OG, Arias ML (2010) Diarrheagenic *Escherichia coli* in children from Costa Rica. Am J Trop Med Hyg 83:292–297

Petty NK, Ben Zakour NL, Stanton-Cook M, Skippington E, Totsika M, Forde BM, Phan M-D, Gomes Moriel D, Peters KM, Davies M, Rogers BA, Dougan G, Rodriguez-Baño J, Pascual A, Pitout JDD, Upton M, Paterson DL, Walsh TR, Schembri MA, Beatson SA (2014) Global dissemination of a multidrug resistant *Escherichia coli* clone. Proc Natl Acad Sci U S A 111:5694–5699

Philippon A, Labia R, Jacoby G (1989) Extended-spectrum beta-lactamases. Antimicrob Agents Chemother 33:1131–1136

Piazza A, Caltagirone M, Bitar I, Nucleo E, Spalla M, Fogato E, D'Angelo R, Pagani L, Migliavacca R (2016) Emergence of *Escherichia coli* sequence Type 131 (ST131) and ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a long-term care and rehabilitation facility (LTCRF) in Northern Italy. Adv Exp Med Biol 901:77–89

Pietsch M, Eller C, Wendt C, Holfelder M, Falgenhauer L, Fruth A, Grössl T, Leistner R, Valenza G, Werner G, Pfeifer Y; RESET Study Group. Molecular characterisation of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates from hospital and ambulatory patients in Germany. Vet Microbiol. 2015 Nov 24. pii: S0378-1135(15)30097-3. doi:10.1016/j.vetmic.2015.11.028. [Epub ahead of print]

Platell JL, Cobbold RN, Johnson JR, Heisig A, Heisig P, Clabots C, Kuskowski MA, Trott DJ (2011) Commonality among fluoroquinolone-resistant sequence type ST131 extraintestinal *Escherichia coli* isolates from humans and companion animals in Australia. Antimicrob Agents Chemother 55:3782–3787

Price LB, Johnson JR, Aziz M, Clabots C, Johnston B, Tchesnokova V, Nordstrom L, Billig M, Chattopadhyay S, Stegger M, Andersen PS, Pearson T, Riddell K, Rogers P, Scholes D, Kahl B, Keim P, Sokurenko EV (2013) The epidemic of extended-spectrum-β-lactamase-producing *Escherichia coli* ST131 is driven by a single highly pathogenic subclone, H30-Rx. MBio 4:e00377-313

Reyna-Flores F, Barrios H, Garza-Ramos U, Sánchez-Pérez A, Rojas-Moreno T, Uribe-Salas FJ, Fagundo-Sierra R, Silva-Sanchez J (2013) Molecular epidemiology of *Escherichia coli* O25b-ST131 isolates causing community-acquired UTIs in Mexico. Diagn Microbiol Infect Dis 76:396–398

Riveros M, Riccobono E, Durand D, Mosquito S, Ruiz J, Rossolini GM, Ochoa TJ, Pallecchi L (2012) Plasmid-mediated quinolone resistance genes in enteroaggregative *Escherichia coli* from infants in Lima, Peru. Int J Antimicrob Agents 39:540–542

Rodríguez MM, Power P, Radice M, Vay C, Famiglietti A, Galleni M, Ayala JA, Gutkind G (2004) Chromosome-encoded CTX-M-3 from Kluyvera ascorbata: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. Antimicrob Agents Chemother 48:4895–4897

Rogers BA, Sidjabat HE, Paterson DL (2011) *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. J Antimicrob Chemother 66:1–14

Scherz G, Stahl J, Glünder G, Kietzmann M (2014) Effects of carry-over of fluoroquinolones on the susceptibility of commensal *Escherichia coli* in the intestinal microbiota of poultry. Berl Munch Tierarztl Wochenschr 127:478–485

Shakir SM, Goldbeck JM, Robison D, Eckerd AM, Chavez-Bueno S (2014) Genotypic and phenotypic characterization of invasive neonatal *Escherichia coli* clinical isolates. Am J Perinatol 31:975–982

Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A (2009) Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 22:664–689

Suwantarat N, Rudin SD, Marshall SH, Hujer AM, Perez F, Hujer KM, Domitrovic TNJ, Dumford DM, Donskey CJ, Bonomo RA (2014) Infections caused by fluoroquinolone-resistant *Escherichia coli* following transrectal ultrasound-guided biopsy of the prostate. J Glob Antimicrob Resist 2:71–76

Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, McDermott PF (2012) Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. Emerging Infect Dis 18:741–749

Teichmann A, de Cunha Agra HN, Nunes L de S, da Rocha MP, Renner JDP, Possuelo LG, Carneiro M, Rieger A, Benitez LB, Valim AR de M (2014) Antibiotic resistance and detection of the sul2 gene in urinary isolates of *Escherichia coli* in patients from Brazil. J Infect Dev Ctries 8:39–43

Valenza G, Nickel S, Pfeifer Y, Pietsch M, Voigtländer E, Lehner-Reindl V, Höller C. Prevalence and genetic diversity of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in nursing homes in Bavaria, Germany. Vet Microbiol. 2015 Oct 19. pii: S0378-1135(15)30048-1. doi: 10.1016/j.vetmic.2015.10.008. [Epub ahead of print]

Vélez Echeverri C, Serna-Higuita LM, Serrano AK, Ochoa-García C, Rojas Rosas L, María Bedoya A, Suárez M, Hincapié C, Henao A, Ortiz D, Vanegas JJ, Zuleta JJ, Espinal D (2014) Resistance profile for pathogens causing urinary tract infection in a pediatric population, and antibiotic treatment response at a university hospital, 2010–2011. Colomb Med 45:39–44

Villar HE, Aubert V, Baserni MN, Jugo MB (2013) Maternal carriage of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in Argentina. J Chemother 25:324–327

Weissman SJ, Hansen NI, Zaterka-Baxter K, Higgins RD, Stoll BJ. Emergence of Antibiotic Resistance-Associated Clones Among *Escherichia coli* Recovered From Newborns With Early-Onset Sepsis and Meningitis in the United States, 2008-2009. J Pediatric Infect Dis Soc. 2015 Mar 31. pii: piv013. [Epub ahead of print]

Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MCJ, Ochman H, Achtman M (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 60:1136–1151

Wirtz VJ, Dreser A, Gonzales R (2010) Trends in antibiotic utilization in eight Latin American countries, 1997–2007. Rev Panam Salud Publica 27:219–225

World Health Organization (2014) Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva, Switzerland

World Health Organization (2015) Worldwide country situation analysis: response to antimicrobial resistance. Geneva, Switzerland

Chapter 14
Interactions of Pathogenic *Escherichia coli* with Host Receptors

Mauricio J. Farfán and Jorge A. Girón

Summary Each *Escherichia coli* pathotype has a unique way to interact with its host. While some of the adhesins that mediate cell adherence and bacteria-to-bacteria interactions are shared among these different categories of pathogenic *E. coli*, other adhesins are pathotype specific. This implies that there are common and unique receptors recognized by this myriad of *E. coli* adhesins, which ultimately determine what host (human, animal, or plant), tissue, or cell type they are colonizing. Notably, both commensal and pathogenic *E. coli* adhere to the gut mucus layer covering and protecting epithelial cells. This is a prerequisite for colonization of the epithelium and establishment of disease. It is then the interaction between surface adhesins and their cognate surface-exposed receptors that determines tropism, unravels mechanisms of pathogenesis, and triggers activation of the local immune responses. Despite our knowledge on the mechanisms of adherence of some pathogenic *E. coli*, much effort is still needed in identifying the eukaryotic receptor counterparts. The most current knowledge on the nature of the receptors involved in the *E. coli*–host interaction is reviewed here.

M.J. Farfán (✉)
Departamento de Pediatría, Facultad de Medicina, Universidad de Chile, Hospital Dr. Luis Calvo Mackenna, Santiago, Chile
e-mail: mfarfan@med.uchile.cl

J.A. Girón
Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

University of Virginia, Puebla and Charlottesville, Mexico, VA, USA
e-mail: jagiron@yahoo.com

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_14

1 *E. coli*–Host Interactions

For commensal or any pathogenic *E. coli*, the ability to detect host signals and interact efficiently with host cells is a prerequisite for successful colonization of the gut mucosa and/or establishment of disease. Immediately after entering the host, the bacteria must face the lethal effect of the low pH of the gastric mucosa. Therefore, pH-resistant bacteria, such as Shiga toxin-producing *E. coli* (STEC) and enteroinvasive *E. coli* (EIEC) require lower doses of infection (e.g., 10–50 organisms) than pH-sensitive *E. coli*, such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC), which require over 10^7 bacteria to infect the host. The low acidity of the stomach signals the bacteria to up-regulate necessary genes in preparation for the upcoming encounter with bile salts and other host signals present in the small bowel. For example, virulence genes in ETEC and EPEC required for toxicity and adherence may be turned on by these signals in preparation for colonization of this anatomical site (Fig. 14.1). EHEC and EAEC, on the other hand, transiently travel through the lumen of the small bowel to reach their site of colonization in the large bowel (Kaper et al. 2004) Once there, these bacteria face host-specific factors that prevail in their colonization niche, such as an anaerobic atmosphere, an intense metabolic activity of digestion of nutrients and adsorption, the intestinal microbiota, and an alert humoral and cellular immune system that is prepared to attack the intruding bacteria (Sommer and Backhed 2013).

What determines whether a particular organism will colonize a host niche is in great part the presence of an array of host-cell receptors, which come in different

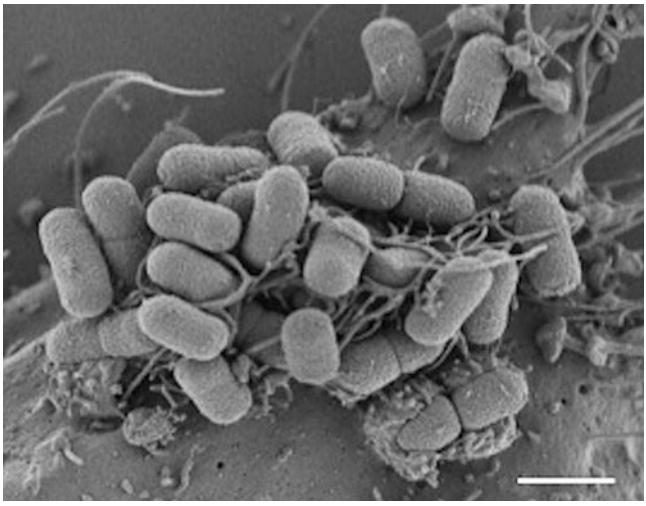

Fig. 14.1 EPEC O127:H6 strain E2348/69 forming localized adherence to a cultured HeLa cell. Several types of interactions occur in this event: (1) Bacteria attaching intimately to the cell surface inducing the formation of pedestals where the bacteria sit atop (*bottom right*). (2) Bacteria tethered through thick cellular pseudopod-like protrusions. (3) Bacteria-to-bacteria interactions through thin and thick pili structures such as ECP and BFP, respectively. Bar, 3 μm

forms and structures. These receptors may be present only on the surface of certain cell types; for example, forming part of the mosaic of surface-exposed molecules on the enterocytes, or secreted in the mucus layer, bathing the epithelium or in the extracellular matrix (EM) of the basal membrane (McGuckin et al. 2011).

Several families of eukaryotic surface molecules can be targets of pathogenic bacteria for binding and colonization including mucins, proteoglycans, cholesterol, membrane integral proteins, carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), toll-like receptors (TLRs), signaling lymphocytic activation molecule family (SLAMF), integrins, and EM proteins. While host cell receptors are widely distributed in different tissues, they present at the same time certain high degree of variability that enables the tropism for adherence to certain cell types and tissues (Juge 2012). One should be mindful that these receptors play a biological function in the host and do not necessarily exist to serve bacterial pathogens. Pathogenic bacteria have engineered their receptors ligands (adhesins) to recognize these cellular receptors for their benefit. Certain pathogenic *E. coli* strains actually produce and inject their own receptor on host cells. Such is the case of the translocated intimin receptor (Tir), a bacterial 90 kDa-protein that is injected into host cells by the type-3 secretion system of EPEC and enterohemorrhagic *E. coli* (EHEC) (Fig. 14.2). Once inserted and displayed on the host cell membrane, Tir serves as the receptor for the bacterial outer membrane protein called Intimin to produce intimate cell attachment (Kenny et al. 1997). Pathogenic *E. coli* strains display a large capacity to adapt their fimbrial adhesins to diverse ecological niches via charge-driven interactions, congruent with binding to mucosal surfaces displaying an acidic gradient along the intestinal tract (Waksman and Hultgren 2009).

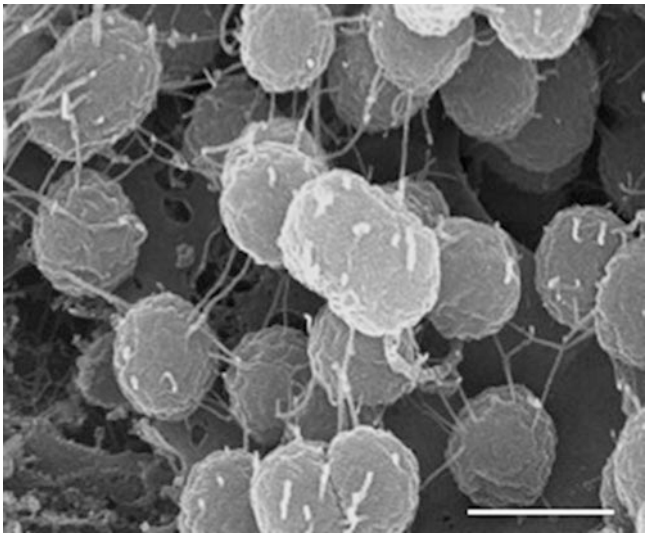

Fig. 14.2 EHEC O157:H7 strain EDL933 attaching to cultured HeLa cells. The bacteria associate with the cell membrane and to each other through peritrichous fibrillar structures that extend out of the bacterial surface. Bar, 1 μm

2 The Intestinal Mucus Barrier

The human intestine is in fact a complex ecosystem where the interrelationship between the microbiota, nutrients, and host epithelial cells has a significant impact upon health and establishment of disease. Gastrointestinal epithelial cells are tightly linked via intracellular junctions that form a contiguous barrier, which is resistant to microbial passage (McGuckin et al. 2011; Juge 2012). The intestinal barrier to infection is constituted by secreted mucus, the apical glycocalyx, and epithelial tight junctions. All along the gastrointestinal tract, a thick mucus layer covers the underlying mucosa, which protects it from harmful organisms present in the lumen content or from incoming enteric pathogens. Thus, the intestinal mucus represents the first line of defense in gut epithelium against dangerous bacteria. Two layers with different composition and function can be distinguished in the gut mucus. The outer loose section of the mucus layer provides a sanctuary for the natural microbiota constituted by thousands of different bacterial species, which play important roles in the physiology and homeostasis of the intestine. The inner mucus layer is firmly attached to the epithelial lining and protects it from possible microbial intruders (McGuckin et al. 2011; Juge 2012).

This biochemically complex gel varies in thickness with the region of the gastrointestinal tract, being thicker in the colon and the rectum. The gastrointestinal tract's mucus is rich in glycoproteins, antimicrobial peptides (e.g., β-defensins), immunoglobulins, lipids, electrolytes, and water. The viscous nature of mucus makes it capable of aggregating and removing microorganisms. The importance of the mucus barrier is underlined by recent reports that mice deficient in MUC2 develop severe, life-threatening disease when infected with the attaching and effacing pathogen *Citrobacter rodentium*. These mice also develop spontaneous intestinal inflammation, consistent with the previously demonstrated nonphysiological exposure of the commensal microbiota to the epithelium when mucus is depleted (McGuckin et al. 2011; Juge 2012).

3 Mucins

The major structural components of the mucus layers are high-molecular-weight proline-, threonine-, and serine-rich glycoproteins called "mucins." These amino acidic residues are heavily glycosylated producing a myriad of oligomeric mucins that provide specificity and programmed function to host cells. There are two types of mucins: membrane-bound and secreted mucins. All mucosal epithelial cells produce and display surface-exposed mucins. These are transmembrane glycoproteins characterized by a cytoplasmic domain involved in signal transduction and an extensively O-glycosylated extracellular domain that may reach up to 800 nm away from the cell surface, which is important in cell adhesion. The outer surface-exposed portion of the glycosylated mucins can be secreted or

shed from the cell and it is thought that this could represent a mechanism to distract bacterial pathogens consequently limiting their access to their target receptors on the epithelium.

The intestine produces membrane-bound mucins: MUC1, MUC3, MUC4, MUC12, MUC13, and MUC17; and secreted mucins: MUC2, MUC5B, MUC5AC, and MUC6 (Juge 2012; Etzold and Juge 2014). The main mucins in the stomach are MUC1, MUC5AC, and MUC6, while in the small intestine and colon, MUC2 is the major component of the mucus layer. More than 100 complex oligosaccharides (mono-, di-, or trisialylated) can be found in colonic MUC2 mucin. This complex diversity of colon glycans is relatively conserved between individuals and highlights the biological importance of these glycans in dictating, through ligand-receptor binding, which bacterial species will conform the commensal microbiota. Bacterial pathogens have learned to recognize mucin glycans as receptors and even regulate changes in production of mucins and their glycosylation. A large body of evidence supports the role of intestinal mucins in maintenance of the gut homeostasis, protecting the host from intruding and invasive pathogens, and regulating immune responses. For example, the lack of MUC2 in mice leads to spontaneous colitis, intestinal inflammation, and development of colorectal cancer (Juge 2012).

4 Bacterial Strategies to Overcome the Gut Mucus Barrier

Enteric pathogens have evolved a wide range of specific strategies to either penetrate or circumvent the secreted and cellular barriers to infection. These strategies include mechanisms allowing efficient penetration of the mucus by producing enzymes that degrade mucus components, through production of flagella that mediates swimming across the dense mucus, using pathways that allow evasion of the barrier, and by disruption of the cells that produce such barrier. Intestinal *E. coli* are capable of swimming across the mucus layer using flagella to reach their target sites on epithelial cells for efficient delivery of toxins and effectors through any of the secretion systems so far described (see Chap. 10). Pathogens with disturbed flagellar function have reduced pathogenicity, underlining the importance of motility in disease. It should be recognized that flagella are also involved in adhesion and activation of immune responses through recognition of TLR-5. Furthermore, some pathogens can alter the integrity of the mucus, affecting its viscoelasticity to their benefit (Giron et al. 2002).

EAEC is a good example of an organism that induces mucus production in the colon favoring attachment and formation of biofilms, a mechanism that has been associated with the characteristic mucoid and persistent diarrhea that distinguishes this pathogen from other diarrheagenic *E. coli* (DEC) strains (Nataro and Kaper 1998).

Many microorganisms have evolved enzymes to degrade mucus. These enzymes include glycosidases that breakdown mucin oligosaccharides, exposing the mucin peptide backbone to proteases, while also removing decoy carbohydrates from

microbial adhesins. Proteolytic cleavage of mucins causes disassembly of the oligomerized mucin macromolecules, resulting in substantially diminished mucus viscosity, dispersal of the mucus, and diffusion and dilution of antimicrobial molecules (McGuckin et al. 2011). For example, EAEC has the mucolytic activity that is required for translocation through mucin-containing gels in the intestine. It produces a mucinase called Pic (Protein involved in colonization), which targets O-glycosylated residues on surface-exposed mucins in different cell types (Harrington et al. 2009; Ayala-Lujan et al. 2014).

EHEC produces StcE, a zinc metalloprotease/mucinase with activity on the intestinal mucin MUC7. It was suggested that this enzyme contributes to intimate adherence of this organism and also functions as an anti-inflammatory molecule, by localizing complement regulator C1-INH to cell membranes (Grys et al. 2006).

A highly conserved metalloprotease encoded by the chromosomal gene *yghJ* was recently reported to influence the ability of ETEC to colonize the small intestine, by degrading the major MUC2 and MUC3 mucins. This gene is also widely spread in other enteric pathogens including *Vibrio cholerae* and other DECs (Luo et al. 2014).

The membrane-bound mucin MUC17 is highly expressed on the apical surface of intestinal epithelia and is thought to play a role in epithelial restitution, in maintaining epithelial barrier function, and protection of the mucosa against luminal pathogens. Reduction of endogenous MUC17 is associated with increased permeability, inducible nitric oxide synthase, and cyclooxygenase 2 induction, as well as enhanced bacterial invasion in response to EIEC exposure, while bacterial adhesion is not affected. These data suggest that MUC17 plays a role in attachment and invasion of EIEC in colonic cell lines and in maintaining a normal epithelial barrier function (Resta-Lenert et al. 2011).

Mucus degradation is not limited to pathogens, as some commensal intestinal bacteria are also mucolytic, and can use mucin glycoproteins as an energy source and also to provide substrates for other nonpathogenic bacteria in the outer mucus layer (McGuckin et al. 2011).

5 *E. coli* Mucin Glycan Receptors

Both commensal and pathogenic *E. coli* compete for binding sites on gastrointestinal mucus. While a number of enteric pathogens interact with mucins at different levels in the gut mucosa, as a prerequisite for colonization, not much is known about the types of mucins that function as receptors for most *E. coli* pathotypes and what adhesins are involved in this process. A few studies described later highlight the importance of mucins in the attachment or anti-inflammatory activity of pathogenic *E. coli* molecules. EAEC binds to intestinal mucins, a property associated with biofilm formation in the colonic outer layer mucus. These bacteria produce Pic that recognizes O-glycosylated serine in mucins (Henderson et al. 1999; Ayala-Lujan et al. 2014) and was shown to promote colonization in a mouse model of

infection (Harrington et al. 2009). The role of flagella in the binding of EPEC and EHEC to host receptors such as mucins and bovine mucus has been documented (Giron et al. 2002; Erdem et al. 2007).

Although many fimbrial and nonfimbrial adhesins have been identified in human pathogenic *E. coli*, the structure of the receptors for only a handful of bacterial fimbrial adhesins is known. The FimH tip adhesin of the type 1 fimbriae is the most widely studied glycan-recognizing protein adhesin with specificity for cellular structures containing α-D-mannosylated proteins and mannose in different conformations leading to different tissue tropism (Sokurenko et al. 1992). The recognition of mannose-rich uroplakin on bladder epithelial cells by uropathogenic *E. coli* (UPEC)'s type 1 fimbriae is a mechanism of colonization of the urinary tract (Kaper et al. 2004). The P fimbriae tip adhesin PapD of UPEC recognizes α-D-galactopyranosyl-(1,4)-β-D-galactopyranoside receptor epitope present on erythrocytes and kidney epithelial cells favoring colonization and production of pyelonephritis (Lindberg et al. 1987). The F17G tip adhesin of the F17 fimbriae of animal ETEC binds to glycoprotein glycan receptors and recognizes terminal *N*-acetyl-glucosamine on intestinal mucins (Mouricout et al. 1995; Lonardi et al. 2013). The F4 fimbriae of animal ETEC recognizes porcine intestinal glycosphingolipids (Coddens et al. 2011). The CS6 fibrillae of human ETEC recognizes the sulfatide (SO3-3Gal β1Cer) glycosphingolipid (Jansson et al. 2009). Arabinans present in plant cell walls function as receptors for the EcpD tip adhesin-mediated adherence of ECP-producing *E. coli* (Rossez et al. 2014). However, the nature of the mammalian cell receptor for ECP is unknown. Most recently, it was reported that CfaE, the tip adhesin of the CFA/I fimbriae, binds to asialo-GM1 on Caco2 cells and erythrocytes demonstrating that asialo-glycosphingolipids are implicated as receptors for this important pilus of ETEC and in mediating binding and colonization of intestinal epithelial cells (Madhavan et al. 2016). The alpha bundlin of the bundle-forming pilus (BFP) of EPEC possesses lectin-like properties that are responsible for N-acetyllactosamine (LacNAc)-specific initial adherence to host intestinal epithelial cells (Hyland et al. 2008). There is a need for more research on the identification of receptor structures for a large number of *E. coli* adhesins.

6 The Proteoglycans

The proteoglycans (PG) are complex ubiquitous molecules which have a different tissue-depending distribution and composition (Garcia and Gerardo 2014). The basic core of proteoglycans consists of different types of proteins modified with chains of anionic polysaccharides called glycosaminoglycans (GAGs). The GAGs are made up of repeated disaccharide units which can be classified as either heparin/heparan sulfate (glucoronic acid plus N-acetylglucosamine [NAG]), chondroitin/dermatan sulfate (glucoronic acid plus N-acetylgalactosamine), keratan sulfate (glucoronic acid plus NAG), and hyaluronic acid (glucoronic acid plus NAG). Among these molecules, heparan sulfate is the most widespread and physiologically relevant

GAG. Heparan-sulfate-containing PGs have multiple functions, some of them related to the core proteins but most certainly to the GAG chains. It is this structural diversity that allow heparin sulfate PGs to play important roles in many cellular processes, such as organization of the basement membrane structure, regulation of proliferation, cell adhesion and migration, cytoskeleton organization, differentiation and morphogenesis, among others. Several bacterial pathogens, including pathogenic *E. coli*, interact with heparin sulfate PGs to achieve adherence and even invade and disseminate (Duan et al. 2013).

However, bacterial glycans such as lipooligosaccharides and lipopolysaccharides were shown recently to interact with host cell glycans with high affinity demonstrating that glycan–glycan interactions mediate binding of pathogenic bacteria to host cells (Day et al. 2015). The recognition of host glycans by pathogenic *E. coli* glycans has yet to be determined but considering the high degree of LPS variants among the *E. coli*, it wouldn't be surprising to find such interactions.

7 Membrane-Associated Cholesterol in Pathogen–Host Interaction

Pathogen–host interactions involve several key components at the cell surface of both the host and the pathogen. Cholesterol is an essential lipid in higher eukaryotic cell membranes and is unique in terms of the functional role it plays in cellular physiology (Simons and Ikonen 2000; Kumar et al. 2016). This lipid is an important player in the entry of intracellular pathogens. EIEC, like *Shigella*, uses T3SS and its effectors to penetrate enterocytes (Kaper et al. 2004). Whether EIEC uses also cholesterol in lipid rafts to gain access to the host cell cytoplasm is unknown.

8 CEACAMs

The carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) comprise a group of immunoglobulin-related glycoproteins, some of which are found at the surface of epithelial cells (Tchoupa et al. 2014). Several biological functions have been associated to CEACAMs and they may be recognized as receptors by pathogenic and nonpathogenic bacteria. Pathogenic *E. coli* strains such as adherent and invasive *E. coli* (AIEC) and diffuse adherent *E. coli* (DAEC) have been shown to interact with CEACAMs (Berger et al. 2004; Barnich et al. 2007).

AIEC strains have been linked to the pathogenesis of Crohn's Disease (CD) characterized by high levels of inflammation of the gut mucosa, presumably triggered by AIEC and influenced by the composition of the intestinal microbiota. Studies with transgenic mice expressing human CEACAMs demonstrated that the FimH tip adhesin is involved in the recognition of CEACAM6 by AIEC (Carvalho et al.

2009). It has been shown that CD patients have increased levels of CEACAM6 expression, which would explain, at least in part, the association of AIEC with this disease. Further, AIEC adherence to enterocytes obtained from CD patients was blocked with anti-CEACAM6 monoclonal antibody. Similar observations were found when IFN-γ-stimulated Caco-2 cells were transfected with siRNA-blocking CEACAM6 (Barnich et al. 2007). Sequence analysis of *fimH* genes from AIEC and non-AIEC strains revealed that point mutations in *fimH* confer AIEC a significantly higher ability to adhere to CEACAM-expressing intestinal epithelial cells in vivo and in vitro (Dreux et al. 2013). These results suggest that in the evolution of pathogenic AIEC strains the selection of FimH polymorphism was key for AIEC pathogenesis in CD patients.

DAEC strains associated to urinary infections express Afa/Dr adhesins, a family of molecules with receptor specificity for the Dr blood group antigen. These adhesins are responsible for the attachment of the bacteria to the surface of epithelial cells. DAEC adherence to epithelial intestinal cells was blocked using antibodies against CEACAM5 (CD66e) (Guignot et al. 2000). Later, experiments carried out with different cell types expressing CEACAM demonstrated that Afa/Dr adhesins F1845 and AfaE-III have the ability to bind to CEACAM1, CEACAM5, or CEACAM6 (Berger et al. 2004). To date, several members of the Dr adhesins are known to bind to CEACAM receptors (Servin 2014), but the significance of the recognition of these receptors in DAEC pathogenesis still needs further investigation.

9 The Extracellular Matrix: One Target Fits All *E. coli*

The extracellular matrix (EM) is a complex protein structure involved in several biological processes and may be recognized as a receptor by many bacterial pathogens (Patti and Hook 1994). The composition of the EM varies with the tissue; in the case of the gastrointestinal tract, fibronectin, laminin, and type I and IV collagens are the main protein constituents (Dubreuil et al. 2002). Given the abundance and adhesive properties of the EM, several research groups have studied the involvement of EM proteins in the recognition of adhesins of several bacterial pathogens, including pathogenic *E. coli* strains (Abraham et al. 1983; Froman et al. 1984; Castaneda-Roldan et al. 2004; Alteri and Mobley 2007; Wells et al. 2009; Christner et al. 2010; Nallapareddy et al. 2011). Although several *E. coli* strains have the ability to bind to EM proteins, the majority of studies focus on the interaction of DECs with EM proteins.

EAEC adhesion to human intestinal mucosa requires the participation of the aggregative adherence fimbriae (AAFs), which promotes the formation of a biofilm on the epithelial surface (Nataro et al. 1992). EAEC has the ability to bind to different components of the EM such as fibronectin, laminin, and collagen IV, a process dependent on the expression of AAFs. Previous studies have shown that preincubation of cultured intestinal epithelial cells with fibronectin increases the adhesion of EAEC (Farfan et al. 2008). Recent studies focused on elucidating the mechanism of

Table 14.1 *E. coli* adhesins recognizing EM proteins

Adhesin	*E. coli* type	Ligand	Reference
AAF fimbria	EAEC	Fn, Lm, CIV	Farfan et al. (2008), Konar et al. (2012)
P fimbriae	UPEC	Fn	Westerlund et al. (1993), Roberts et al. (1997)
Curli	All	Fn, Lm	Olsen et al. (1989), Farfan et al. (2011)
FimH	All	Fn, Lm	Sokurenko et al. (1992), Kukkonen et al. (1993)
Bfp fimbriae	EPEC	Fn, Lm, CIV	Giron et al. (1993)
S fimbriae	MAEC	Fn	Saren et al. (1999)
Tsh autotransporter	APEC	Fn, CIV	Kostakioti and Stathopoulos (2004)
Flagella	EPEC, STEC	Fn, Lm	Erdem et al. (2007)
HCP	STEC	Fn, Lm	Xicohtencatl-Cortes et al. (2009)
ELF fimbriae	STEC	Lm	Samadder et al. (2009)
Ehly hemolysin	EPEC	Fn	Magalhaes et al. (2011)
Lpf fimbriae	STEC	Fn, Lm, CIV	Farfan et al. (2011)
GroEL	aEPEC	Fn	Moraes et al. (2015)
CS6	ETEC	Fn	Ghosal et al. (2009)

Fn, Fibronectin; Lm, Laminin; CIV, Collagen IV

EAEC adherence mediated by AAF-fibronectin have shown that the interaction between these two proteins involves electrostatic interactions attributed to the presence of basic residues in the AAF (Berry et al. 2014).

Similar to EAEC, several pathogenic *E. coli* adhesins have the ability to bind to EM proteins (Table 14.1). Thus, it is not surprising that a particular adhesin has the ability to bind to one or several EM proteins. However, one of the main questions regarding the ability of *E. coli* to adhere to these proteins is the biological significance of this binding. Future studies should address the importance of this interaction and its implication in the establishment of disease.

10 Toll-Like Receptors

Toll-like receptors (TLR) are a family of transmembrane immune receptors that trigger an inflammatory cascade reaction in response to pathogen-associated molecular patterns (PAMPs). Currently, there are ten TLRs described and agonists for nine of them have been determined (Takeda and Akira 2004). This family of proteins constitutes a network that functions as an alarm system that alerts the immune system of the presence of microorganisms. For example, some TLRs recognize

viruses, other recognize Gram-positive or Gram-negative bacteria, and so on. The LPS and flagellin are classical bacterial agonists that bind to TLR4 and TLR5, respectively, although there are other factors that participate in the activation of TLR by bacteria. Upon activation of TLRs, a signal cascade is initiated which results in the activation of expression of genes encoding proinflammatory molecules. The type 1 and P fimbriae of UPEC trigger mucosal inflammation through the stimulation of a TLR4-dependent pathway (Frendeus et al. 2001; Mian et al. 2010). The interaction of a particular bacterial adhesin with the surface of epithelial cells promotes the engagement of bacterial TLR agonists, such as LPS, triggering intracellular cascades involved in the inflammatory response originated as a consequence of the infection process.

11 SLAMF Receptors

The signaling lymphocytic activation molecule family (SLAMF) is comprised of nine surface glycoproteins receptors expressed mostly in hematopoietic cells. Most SLAMF receptors are self-ligands with signaling motifs, which function in cell–cell communication and can also bind bacterial structures (van Driel et al. 2016). The *E. coli* outer membrane porin C (OmpC) and OmpF have the ability to bind to SLAMF1 and SLAMF6. The interaction of SLAMF1 with nonpathogenic *E. coli* strains expressing Omps C and F results in a more effective phagocytosis of these bacteria by macrophages (Berger et al. 2010). Similarly, *E. coli* expressing FimH binds to SLAMF2 and antibodies against this receptor inhibit the phagocytosis of the *E. coli* by mast cells and macrophages, a process mediated by cellular caveolae (Shin et al. 2000). Further studies are necessary to elucidate the involvement of SLAMF in the innate and adaptive immune responses to *E. coli*, in order to design new strategy to control the host immune response to *E. coli*.

12 Integrins

Integrins are heterodimeric glycoproteins that are critical for a variety of cell–cell and cell–matrix binding events. Once a particular ligand binds to integrin it triggers a specific signaling pathway depending on the type of integrin that is modulated (Hauck and Ohlsen 2006). Bacteria can bind directly and indirectly to integrin. UPEC's type 1 fimbriae bind to integrins β1 and α3 and to the heterodimers formed between these proteins (Eto et al. 2007). Integrin α5β1 participates in the fibronectin-mediated adherence of EAEC to intestinal cells. Fibronectin-associated bacteria can be recognized by integrin α5β1 through the RGD (Arginine-Glycine-Aspartic acid) region, and this engagement increases the adherence of EAEC to intestinal cells (Izquierdo et al. 2014a).

13 The Mosaic of *E. coli* Adhesins

DEC have interacted with their host(s) for thousands of years and have developed multiple strategies to overcome the bactericidal and inhibitory effects of the resident microbiota and the specific and nonspecific factors of the immune system, as well as to produce an arsenal of surface molecules that allow them to recognize host cell receptors present in mucus layers or embedded in the epithelium. Ultimately, DEC strains display surface proteinaceous structures present as polymerized hair-like filaments called fimbriae or pili, or as nonfimbrial adhesins that attach to the gut mucosal epithelium. Different adhesins can coexist in the same pathogen, all of which may act in concert at different stages during infection to successfully colonize the host. The expression and production of these adherence factors are tightly regulated by complex regulatory networks influenced by niche-specific host factors.

Several different fimbrial and nonfimbrial adhesins have been described for the various *E. coli* pathotypes (Farfan and Torres 2012). While some adhesins are unique to an individual pathogenic category, other adhesins may be present and shared in the various groups. For example, the colonization factors (CFs) of ETEC strains are found only in ETEC, the production of the bundle-forming pilus (BFP) is a phenotypic characteristic of EPEC strains only, and the AAF are found only in EAEC strains (Giron et al. 1991; Kaper et al. 2004; Qadri et al. 2005). On the other hand, genes encoding for expression of the long-polar fimbriae (Lpf) may be found in multiple *E. coli* pathotypes (Torres et al. 2009; Galli et al. 2010).

The genetic core of the *E. coli* may contain 12–16 distinct pili operons, depending on the strain, some of which may be produced under certain growth in vitro conditions (Perna et al. 2001; Pouttu et al. 2001; Rendon et al. 2007). Classic examples of core-encoded fimbriae are type 1 fimbriae and curli. While these pili may or may not be important for or even produced by some pathogenic *E. coli*, in general they confer increased adhesiveness and survival fitness in different niches in or outside the host to the bacteria that produce them. Production of other core-encoded fimbrial structures in EHEC and other pathogenic *E. coli* has been reported (Figs. 14.1 and 14.2). The meningitis-associated temperature-dependent fimbriae (MAT) also called *E. coli* common pilus (ECP) are produced by all pathogenic *E. coli* including human and animal strains and they are important for cell adherence and biofilm formation (Pouttu et al. 2001; Rendon et al. 2007; Avelino et al. 2010; Saldana et al. 2014). Receptor recognition is presumably mediated by the tip adhesin protein EcpD (Garnett et al. 2012). The assembly of the *E. coli* laminin-binding fimbriae (ELF) is driven from the chromosomal *ycbQ* operon and the pilin of the hemorrhagic coli type IV pilus (HCP) is encoded by the *ppdD* gene as demonstrated in EHEC (Samadder et al. 2009).

Several outbreaks of diarrheal disease associated with the consumption of EHEC-tainted agricultural products have been reported worldwide. This strongly indicated that EHEC interacts with different salad leaves and vegetables. The interaction of EHEC with spinach leaves involves the participation of several adhesins including the type3 secretion system, flagella, curli, ECP, and HCP (Saldana et al.

2011). It is not far-fetched to think that these fimbrial adhesins mediate colonization to different cell receptors in different hosts at different stages during infection although some may be host specific.

Bacterial adhesins are not restricted to fimbrial structures. Many surface-exposed, membrane-associated proteins, and even secreted proteins have been proposed as adhesins. These adhesins may be associated with any of the six secretion systems so far identified in bacteria (see Chap. 10). While the primordial function of these proteins may not be to directly mediate adherence, they definitely contribute to host mucosal colonization. We cannot ignore the contribution of polysaccharide-containing structures such as capsules, LPS, glycocalyx, and extracellular polysaccharides to bacterial adherence and biofilm formation on biological and nonbiological surfaces. Therefore, high affinity biomolecular interactions can mediate binding of pathogenic bacteria to host cells. The different fimbrial and nonfimbrial adhesins of the several *E. coli* pathotypes have been recently reviewed elsewhere and will not be individually discussed here (Qadri et al. 2005; Farfan and Torres 2012; Madhavan and Sakellaris 2015) (see Chaps. 1–6, and 9).

14 *E. coli*–Host Interaction Studies in Latin America (LA)

Most of the studies on *coli*–host interaction performed in LA focused on the molecular epidemiology of pathogenic *E. coli*, specifically DEC strains. The main reason that drives the investigation in this research area is the epidemiological importance of diarrheal diseases in LA and the high prevalence of hemolytic uremic syndrome (HUS) associated to STEC infections in certain regions of South America. The majority of these studies focused on the prevalence and characterization of virulence factors in DEC clinical isolates, as well as on the identification of newly discovered adherence factors and their participation in the infection process. These studies have contributed significantly to the understanding of the mechanisms of pathogenicity of DECs and will help to develop new tools to control these infections in LA and the rest of the world. In relation to the mechanism of *E. coli*–host interaction, there are several research groups in LA focused on the identification and characterization of receptors for *E. coli*. A brief description of some of these findings is discussed as follows.

15 Participation of EM Proteins in *E. coli* Adherence

Given the large number of adhesins present in the different pathogenic *E. coli* strains, it is reasonable to state that a subset of them may recognize EM proteins. For example, fibronectin proteins participate in the adherence of EAEC to epithelial cells (Farfan et al. 2008; Konar et al. 2012). Integrin α5β1 is the major integrin involved in the indirect recognition of bacterial pathogens that possess ability to

bind to fibronectin. *Staphylococcus aureus*, for example, binds to epithelial cells in the respiratory tract, using surface fibronectin-binding proteins. The fibronectin in this complex binds to cellular integrin acting as a "molecular bridge" connecting the bacteria to the cell surface. Similarly, EAEC attaches to the epithelial cells through an AAF-fibronectin-integrin binding mechanism (Izquierdo et al. 2014b). In contrast to typical EPEC, the mechanisms of adherence of atypical EPEC (aEPEC) to epithelial cell are poorly characterized. The binding of a clinical isolate of aEPEC (strain BA2103) to EM proteins, including fibronectin, was reported. A proteomic approach employing supernatants obtained from cultures of BA2013 and purified fibronectin allows the identification of several candidates responsible of the binding of this EM protein. Further characterization of these candidates demonstrated that H11 flagellin and GroEL protein were associated to the ability of this aEPEC isolate to bind to fibronectin (Moraes et al. 2015).

16 Stx and Central Nervous System Complications

Argentina holds the highest record worldwide of HUS with an incidence of 17/100,000 cases in children less than 5 years old (Masana et al. 2010). Considering that around 30 % of the affected population manifests central nervous system complications, several research groups had focused on the mechanisms of Stx-mediated HUS pathogenesis. It was shown that the intracellular administration of Stx in rat brains induced the expression of Gb3, the Stx receptor, in neurons and the microscopy analysis detected Stx in neurons that expressed Gb3 (Tironi-Farinati et al. 2010), as described in humans (Obata et al. 2008).

17 Novel Receptors

Keratins or cytokeratins are the largest subgroup of intermediate filament proteins and are important constituents of the cell cytoskeleton. These proteins are indispensable for the mechanical stability and integrity of epithelial cells and tissues. To date, 19 human cytokeratins have been identified and all of them are expressed on epithelial cells. The composition of cytokeratins differs among tissues, but CK8, CK18, and CK19 are common constituents of the intestinal epithelia. Using a proteomic approach, cytokeratin-8 (CK8) was found to be a potential receptor for the AAF/II fimbriae and Pet autotransporter of EAEC in intestinal epithelial cells. Anti-CK8 and *ck8* small interfering RNA (siRNA) reduced the adherence of EAEC strains and blocked the cytotoxic effect induced by Pet to epithelial cells, respectively (Nava-Acosta and Navarro-Garcia 2013; Izquierdo et al. 2014b).

Acknowledgments This work was supported by grants 1120809 and 1160426 from FONDECYT to M.J.F.

References

Abraham SN, Beachey EH, Simpson WA (1983) Adherence of *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* to fibronectin-coated and uncoated epithelial cells. Infect Immun 41:1261–1268

Alteri CJ, Mobley HL (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. Infect Immun 75:2679–2688

Avelino F, Saldana Z, Islam S, Monteiro-Neto V, Dall'Agnol M, Eslava CA, Giron JA (2010) The majority of enteroaggregative *Escherichia coli* strains produce the *E. coli* common pilus when adhering to cultured epithelial cells. Int J Med Microbiol 300:440–448

Ayala-Lujan JL, Vijayakumar V, Gong M, Smith R, Santiago AE, Ruiz-Perez F (2014) Broad spectrum activity of a lectin-like bacterial serine protease family on human leukocytes. PLoS One 9, e107920

Barnich N, Carvalho FA, Glasser AL et al (2007) CEACAM6 acts as a receptor for adherent-invasive *E. coli*, supporting ileal mucosa colonization in Crohn disease. J Clin Invest 117:1566–1574

Berger CN, Billker O, Meyer TF, Servin AL, Kansau I (2004) Differential recognition of members of the carcinoembryonic antigen family by Afa/Dr adhesins of diffusely adhering *Escherichia coli* (Afa/Dr DAEC). Mol Microbiol 52:963–983

Berger SB, Romero X, Ma C et al (2010) SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. Nat Immunol 11:920–927

Berry AA, Yang Y, Pakharukova N et al (2014) Structural insight into host recognition by aggregative adherence fimbriae of enteroaggregative *Escherichia coli*. PLoS Pathog 10, e1004404

Carvalho FA, Barnich N, Sivignon A, Darcha C, Chan CH, Stanners CP, Darfeuille-Michaud A (2009) Crohn's disease adherent-invasive Escherichia coli colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. J Exp Med 206:2179–2189

Castaneda-Roldan EI, Avelino-Flores F, Dall'Agnol M, Freer E, Cedillo L, Dornand J, Giron JA (2004) Adherence of *Brucella* to human epithelial cells and macrophages is mediated by sialic acid residues. Cell Microbiol 6:435–445

Christner M, Franke GC, Schommer NN et al (2010) The giant extracellular matrix-binding protein of *Staphylococcus epidermidis* mediates biofilm accumulation and attachment to fibronectin. Mol Microbiol 75:187–207

Coddens A, Valis E, Benktander J, Angstrom J, Breimer ME, Cox E, Teneberg S (2011) Erythrocyte and porcine intestinal glycosphingolipids recognized by F4 fimbriae of enterotoxigenic *Escherichia coli*. PLoS One 6, e23309

Day CJ, Tran EN, Semchenko EA et al (2015) Glycan:glycan interactions: high affinity biomolecular interactions that can mediate binding of pathogenic bacteria to host cells. Proc Natl Acad Sci U S A 112:E7266–E7275

Dreux N, Denizot J, Martinez-Medina M et al (2013) Point mutations in FimH adhesin of Crohn's disease-associated adherent-invasive *Escherichia coli* enhance intestinal inflammatory response. PLoS Pathog 9, e1003141

Duan R, Chen X, Wang F, Zhang T, Ling P (2013) Oral administration of heparin or heparosan increases the *Lactobacillus* population in gut microbiota of rats. Carbohydr Polym 94:100–105

Dubreuil JD, Giudice GD, Rappuoli R (2002) *Helicobacter pylori* interactions with host serum and extracellular matrix proteins: potential role in the infectious process. Microbiol Mol Biol Rev 66:617–629

Erdem AL, Avelino F, Xicohtencatl-Cortes J, Giron JA (2007) Host protein binding and adhesive properties of H6 and H7 flagella of attaching and effacing *Escherichia coli*. J Bacteriol 189:7426–7435

Eto DS, Jones TA, Sundsbak JL, Mulvey MA (2007) Integrin-mediated host cell invasion by type 1-piliated uropathogenic *Escherichia coli*. PLoS Pathog 3, e100

Etzold S, Juge N (2014) Structural insights into bacterial recognition of intestinal mucins. Curr Opin Struct Biol 28:23–31

Farfan MJ, Torres AG (2012) Molecular mechanisms that mediate colonization of Shiga toxin-producing *Escherichia coli* strains. Infect Immun 80:903–913

Farfan MJ, Inman KG, Nataro JP (2008) The major pilin subunit of the AAF/II fimbriae from enteroaggregative *Escherichia coli* mediates binding to extracellular matrix proteins. Infect Immun 76:4378–4384

Farfan MJ, Cantero L, Vidal R, Botkin DJ, Torres AG (2011) Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. Infect Immun 79:3744–3750

Frendeus B, Wachtler C, Hedlund M, Fischer H, Samuelsson P, Svensson M, Svanborg C (2001) *Escherichia coli* P fimbriae utilize the Toll-like receptor 4 pathway for cell activation. Mol Microbiol 40:37–51

Froman G, Switalski LM, Faris A, Wadstrom T, Hook M (1984) Binding of *Escherichia coli* to fibronectin. A mechanism of tissue adherence. J Biol Chem 259:14899–14905

Galli L, Torres AG, Rivas M (2010) Identification of the long polar fimbriae gene variants in the locus of enterocyte effacement-negative Shiga toxin-producing *Escherichia coli* strains isolated from humans and cattle in Argentina. FEMS Microbiol Lett 308:123–129

Garcia JR, Gerardo NM (2014) The symbiont side of symbiosis: do microbes really benefit? Front Microbiol 5:510

Garnett JA, Martinez-Santos VI, Saldana Z et al (2012) Structural insights into the biogenesis and biofilm formation by the *Escherichia coli* common pilus. Proc Natl Acad Sci U S A 109:3950–3955

Ghosal A, Bhowmick R, Banerjee R et al (2009) Characterization and studies of the cellular interaction of native colonization factor CS6 purified from a clinical isolate of enterotoxigenic *Escherichia coli*. Infect Immun 77:2125–2135

Giron JA, Ho AS, Schoolnik GK (1991) An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 254:710–713

Giron JA, Ho AS, Schoolnik GK (1993) Characterization of fimbriae produced by enteropathogenic *Escherichia coli*. J Bacteriol 175:7391–7403

Giron JA, Torres AG, Freer E, Kaper JB (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol Microbiol 44:361–379

Grys TE, Walters LL, Welch RA (2006) Characterization of the StcE protease activity of *Escherichia coli* O157:H7. J Bacteriol 188:4646–4653

Guignot J, Peiffer I, Bernet-Camard MF, Lublin DM, Carnoy C, Moseley SL, Servin AL (2000) Recruitment of CD55 and CD66e brush border-associated glycosylphosphatidylinositol-anchored proteins by members of the Afa/Dr diffusely adhering family of *Escherichia coli* that infect the human polarized intestinal Caco-2/TC7 cells. Infect Immun 68:3554–3563

Harrington SM, Sheikh J, Henderson IR, Ruiz-Perez F, Cohen PS, Nataro JP (2009) The Pic protease of enteroaggregative *Escherichia coli* promotes intestinal colonization and growth in the presence of mucin. Infect Immun 77:2465–2473

Hauck CR, Ohlsen K (2006) Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. Curr Opin Microbiol 9:5–11

Henderson IR, Czeczulin J, Eslava C, Noriega F, Nataro JP (1999) Characterization of pic, a secreted protease of *Shigella flexneri* and enteroaggregative *Escherichia coli*. Infect Immun 67:5587–5596

Hyland RM, Sun J, Griener TP, Mulvey GL, Klassen JS, Donnenberg MS, Armstrong GD (2008) The bundlin pilin protein of enteropathogenic *Escherichia coli* is an N-acetyllactosamine-specific lectin. Cell Microbiol 10:177–187

Izquierdo M, Alvestegui A, Nataro JP, Ruiz-Perez F, Farfan MJ (2014a) Participation of integrin alpha5beta1 in the fibronectin-mediated adherence of enteroaggregative *Escherichia coli* to intestinal cells. Biomed Res Int 2014:781246

Izquierdo M, Navarro-Garcia F, Nava-Acosta R, Nataro JP, Ruiz-Perez F, Farfan MJ (2014b) Identification of cell surface-exposed proteins involved in the fimbria-mediated adherence of enteroaggregative *Escherichia coli* to intestinal cells. Infect Immun 82:1719–1724

Jansson L, Tobias J, Jarefjall C, Lebens M, Svennerholm AM, Teneberg S (2009) Sulfatide recognition by colonization factor antigen CS6 from enterotoxigenic *Escherichia coli*. PLoS One 4, e4487
Juge N (2012) Microbial adhesins to gastrointestinal mucus. Trends Microbiol 20:30–39
Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140
Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB (1997) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91:511–520
Konar M, Sachin O, Priya A, Ghosh S (2012) Identification of key proteins of cultured human intestinal cells involved in interaction with enteroaggregative *Escherichia coli*. FEMS Immunol Med Microbiol 66:177–190
Kostakioti M, Stathopoulos C (2004) Functional analysis of the Tsh autotransporter from an avian pathogenic *Escherichia coli* strain. Infect Immun 72:5548–5554
Kukkonen M, Raunio T, Virkola R et al (1993) Basement membrane carbohydrate as a target for bacterial adhesion: binding of type I fimbriae of *Salmonella enterica* and *Escherichia coli* to laminin. Mol Microbiol 7:229–237
Kumar GA, Jafurulla M, Chattopadhyay A (2016) The membrane as the gatekeeper of infection: cholesterol in host-pathogen interaction. Chem Phys Lipids. doi:10.1016/j.chemphyslip.2016.02.007
Lindberg F, Lund B, Johansson L, Normark S (1987) Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. Nature 328:84–87
Lonardi E, Moonens K, Buts L et al (2013) Structural sampling of glycan interaction profiles reveals mucosal receptors for fimbrial adhesins of enterotoxigenic *Escherichia coli*. Biology 2:894–917
Luo Q, Kumar P, Vickers TJ et al (2014) Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect Immun 82:509–521
Madhavan TP, Sakellaris H (2015) Colonization factors of enterotoxigenic *Escherichia coli*. Adv Appl Microbiol 90:155–197
Madhavan TP, Riches JD, Scanlon MJ, Ulett GC, Sakellaris H (2016) Binding of CFA/I Pili of enterotoxigenic *Escherichia coli* to asialo-GM1 is mediated by the minor pilin, CfaE. Infect Immun 84:1642–1649
Magalhaes CA, Rossato SS, Barbosa AS, Santos TO, Elias WP, Sircili MP, Piazza RM (2011) The ability of haemolysins expressed by atypical enteropathogenic *Escherichia coli* to bind to extracellular matrix components. Mem Inst Oswaldo Cruz 106:146–152
Masana MO, Leotta GA, Del Castillo LL et al (2010) Prevalence, characterization, and genotypic analysis of *Escherichia coli* O157:H7/NM from selected beef exporting abattoirs of Argentina. J Food Prot 73:649–656
McGuckin MA, Linden SK, Sutton P, Florin TH (2011) Mucin dynamics and enteric pathogens. Nat Rev Microbiol 9:265–278
Mian MF, Lauzon NM, Andrews DW, Lichty BD, Ashkar AA (2010) FimH can directly activate human and murine natural killer cells via TLR4. Mol Ther 18:1379–1388
Moraes CT, Polatto JM, Rossato SS et al (2015) Flagellin and GroEL mediates in vitro binding of an atypical enteropathogenic *Escherichia coli* to cellular fibronectin. BMC Microbiol 15:278
Mouricout M, Milhavet M, Durie C, Grange P (1995) Characterization of glycoprotein glycan receptors for *Escherichia coli* F17 fimbrial lectin. Microb Pathog 18:297–306
Nallapareddy SR, Singh KV, Sillanpaa J, Zhao M, Murray BE (2011) Relative contributions of Ebp Pili and the collagen adhesin ace to host extracellular matrix protein adherence and experimental urinary tract infection by *Enterococcus faecalis* OG1RF. Infect Immun 79:2901–2910
Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201
Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM (1992) Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun 60:2297–2304
Nava-Acosta R, Navarro-Garcia F (2013) Cytokeratin 8 is an epithelial cell receptor for Pet, a cytotoxic serine protease autotransporter of Enterobacteriaceae. MBio 4:e00838-00813

Obata F, Tohyama K, Bonev AD et al (2008) Shiga toxin 2 affects the central nervous system through receptor globotriaosylceramide localized to neurons. J Infect Dis 198:1398–1406
Olsen A, Jonsson A, Normark S (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature 338:652–655
Patti JM, Hook M (1994) Microbial adhesins recognizing extracellular matrix macromolecules. Curr Opin Cell Biol 6:752–758
Perna NT, Plunkett G 3rd, Burland V et al (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature 409:529–533
Pouttu R, Westerlund-Wikstrom B, Lang H et al (2001) matB, a common fimbrillin gene of *Escherichia coli*, expressed in a genetically conserved, virulent clonal group. J Bacteriol 183:4727–4736
Qadri F, Svennerholm AM, Faruque AS, Sack RB (2005) Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 18:465–483
Rendon MA, Saldana Z, Erdem AL et al (2007) Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. Proc Natl Acad Sci U S A 104:10637–10642
Resta-Lenert S, Das S, Batra SK, Ho SB (2011) Muc17 protects intestinal epithelial cells from enteroinvasive *E. coli* infection by promoting epithelial barrier integrity. Am J Physiol Gastrointest Liver Physiol 300:G1144–G1155
Roberts JA, Kaack MB, Baskin G, Marklund BI, Normark S (1997) Epitopes of the P-fimbrial adhesin of *E. coli* cause different urinary tract infections. J Urol 158:1610–1613
Rossez Y, Holmes A, Lodberg-Pedersen H et al (2014) *Escherichia coli* common pilus (ECP) targets arabinosyl residues in plant cell walls to mediate adhesion to fresh produce plants. J Biol Chem 289:34349–34365
Saldana Z, Sanchez E, Xicohtencatl-Cortes J, Puente JL, Giron JA (2011) Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:H7. Front Microbiol 2:119
Saldana Z, De la Cruz MA, Carrillo-Casas EM et al (2014) Production of the *Escherichia coli* common pilus by uropathogenic *E. coli* is associated with adherence to HeLa and HTB-4 cells and invasion of mouse bladder urothelium. PLoS One 9:e101200
Samadder P, Xicohtencatl-Cortes J, Saldana Z, Jordan D, Tarr PI, Kaper JB, Giron JA (2009) The *Escherichia coli* ycbQRST operon encodes fimbriae with laminin-binding and epithelial cell adherence properties in Shiga-toxigenic *E. coli* O157:H7. Environ Microbiol 11:1815–1826
Saren A, Virkola R, Hacker J, Korhonen TK (1999) The cellular form of human fibronectin as an adhesion target for the S fimbriae of meningitis-associated *Escherichia coli*. Infect Immun 67:2671–2676
Servin AL (2014) Pathogenesis of human diffusely adhering *Escherichia coli* expressing Afa/Dr adhesins (Afa/Dr DAEC): current insights and future challenges. Clin Microbiol Rev 27:823–869
Shin JS, Gao Z, Abraham SN (2000) Involvement of cellular caveolae in bacterial entry into mast cells. Science 289:785–788
Simons K, Ikonen E (2000) How cells handle cholesterol. Science 290:1721–1726
Sokurenko EV, Courtney HS, Abraham SN, Klemm P, Hasty DL (1992) Functional heterogeneity of type 1 fimbriae of *Escherichia coli*. Infect Immun 60:4709–4719
Sommer F, Backhed F (2013) The gut microbiota—masters of host development and physiology. Nat Rev Microbiol 11:227–238
Takeda K, Akira S (2004) Microbial recognition by toll-like receptors. J Dermatol Sci 34:73–82
Tchoupa AK, Schuhmacher T, Hauck CR (2014) Signaling by epithelial members of the CEACAM family—mucosal docking sites for pathogenic bacteria. Cell Commun Signal 12:27
Tironi-Farinati C, Loidl CF, Boccoli J, Parma Y, Fernandez-Miyakawa ME, Goldstein J (2010) Intracerebroventricular Shiga toxin 2 increases the expression of its receptor globotriaosylceramide and causes dendritic abnormalities. J Neuroimmunol 222:48–61

Torres AG, Blanco M, Valenzuela P et al (2009) Genes related to long polar fimbriae of pathogenic *Escherichia coli* strains as reliable markers to identify virulent isolates. J Clin Microbiol 47:2442–2451

van Driel BJ, Liao G, Engel P, Terhorst C (2016) Responses to microbial challenges by SLAMF receptors. Front Immunol 7:4

Waksman G, Hultgren SJ (2009) Structural biology of the chaperone-usher pathway of pilus biogenesis. Nat Rev Microbiol 7:765–774

Wells TJ, McNeilly TN, Totsika M, Mahajan A, Gally DL, Schembri MA (2009) The *Escherichia coli* O157:H7 EhaB autotransporter protein binds to laminin and collagen I and induces a serum IgA response in O157:H7 challenged cattle. Environ Microbiol 11:1803–1814

Westerlund B, Van Die I, Hoekstra W, Virkola R, Korhonen TK (1993) P fimbriae of uropathogenic *Escherichia coli* as multifunctional adherence organelles. Zentralbl Bakteriol 278:229–237

Xicohtencatl-Cortes J, Monteiro-Neto V, Saldana Z, Ledesma MA, Puente JL, Giron JA (2009) The type 4 pili of enterohemorrhagic *Escherichia coli* O157:H7 are multipurpose structures with pathogenic attributes. J Bacteriol 191:411–421

Chapter 15
Human Diarrheal Infections: Diagnosis of Diarrheagenic *Escherichia coli* Pathotypes

Elizabeth Miliwebsky, Felipe Schelotto, Gustavo Varela, Daniela Luz, Isabel Chinen, and Roxane M.F. Piazza

Summary Diarrheagenic *E. coli* as a pathotype deserves special attention since it is responsible for up to 30–40 % of acute diarrhea episodes in children in developing countries; these bacteria are being recognized as important pathogens even in the developed world. However, the importance of diarrheagenic *E. coli* is underestimated due to currently available diagnostic methods with limited applications in routine laboratories, especially in developing countries. Several approaches have been described and applied for the diagnosis of diarrheagenic *E. coli* strains. Thus, this chapter presents general concepts about *E. coli* diagnosis and recent advances in isolation, screening, and identification procedures, in which mass spectrometry and whole genome sequencing are also discussed. It is important to mention that the method of choice will depend on the facilities available in each laboratory, taking into account the sensitivity, specificity, reproducibility, reliability, cost, infrastructural resources, and technical skills required for each method. Apart from diagnostic methods, an overall picture of advances in the Americas in the diagnosis of pathogenic *E. coli* strains is also included, considering the differences between countries regarding the epidemiology of diarrhea caused by *E. coli* strains.

E. Miliwebsky • I. Chinen
Servicio Fisiopatogenia, Departamento de Bacteriología, Instituto Nacional de Enfermedades Infecciosas “Carlos G. Malbrán”, Buenos Aires, Argentina
e-mail: emiliwebsky@gmail.com; ichinen@anlis.gov.ar

F. Schelotto • G. Varela
Departamento de Bacteriología y Virología, Facultad de Medicina, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay
e-mail: felipe@higiene.edu.uy; gvarela@higiene.edu.uy

D. Luz • R.M.F. Piazza (✉)
Laboratório de Bacteriologia, Instituto Butantan, São Paulo, SP, Brazil
e-mail: daniedaluz@yahoo.com.br; roxane.piazza@butantan.gov.br

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6_15

1 General Concept About *Escherichia coli* Diagnosis

Diarrheagenic *Escherichia coli* (DEC) is a bacterial group that deserves special attention because it is responsible for up to 30–40 % of acute diarrhea episodes in children in developing countries (Torres et al. 2001; Patzi-Vargas et al. 2015); these bacteria are also recognized as important pathogens in the developed world. DEC comprise several overlapping categories or pathotypes: enteropathogenic *E. coli* (EPEC), Shiga-toxin-producing *E. coli* (STEC), and its subgroup enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and enteroinvasive *E. coli* (EIEC). Pathogenic properties of other pathotypes and their significance diffuse-adherent *E. coli* (DAEC) or adherent-invasive *E. coli* (AIEC) are still being debated. The relative importance of each pathotype is different depending on the country or region analyzed and should be periodically reviewed.

In general terms, the procedures used for the etiological diagnosis of diarrheal episodes (also frequently used for DEC characterization) can be grouped into classic microbiological selective cultures and biochemical identification procedures; bioassays, immunoassays: enzyme immunoassay (EIA), immunochromatographic tests and DNA-based assays, including nucleic acid amplification techniques (NAAT) such as PCR, qPCR, LAMP (loop-mediated isothermal amplification), TaqMan® Array Card (TAC) platform, or DNA hybridization tests.

In routine microbiology laboratories, all *E. coli* colonies obtained from primary isolation plates can be screened using the classical EPEC serogroup antisera O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (WHO 1987). *E. coli* colonies detected as sorbitol negative on SMAC plates can be tested against O157 antiserum and all other sorbitol fermenting and nonfermenting *E. coli* colonies can be screened for O26, O1O3, O111, and O145 (some of the most common STEC serogroups associated with human infections worldwide) (Scheutz and Strockbine 2005). In addition, all *E. coli* colonies classified as lysine decarboxylase negative, nonmotile, or lactose negative can be screened for the classical EIEC serogroups O28ac, O29, O112, O124, O136, O143, O144, O152, O159, O164, O169, and O173 (Ewing 1986; Ørskov and Ørskov 1992), since most EIEC strains exhibit these characteristics.

A practical and easy test to perform is the slide agglutination test, whose main advantage is the availability of commercial sera. However, the disadvantages of this method are the EPEC serogroups' heterogeneity, which can comprise categories other than EPEC (e.g., O26 and O111 also belong to the STEC category), the inability to distinguish typical (tEPEC) from atypical EPEC (aEPEC) within these serogroups, and the occurrence of EPEC strains belonging to serogroups other than the classical EPEC. Misdiagnosis can also occur due to some atypical EIEC strains [lactose$^+$ (Lac$^+$), gas$^+$ or lysine decarboxylase$^+$ (LDC$^+$) indole$^-$, or motile] (Escher et al. 2014), newly recognized EIEC serogroups, and descriptions different from those mentioned earlier (Ørskov et al. 1991). All *E. coli* isolates screened by this method and belonging to EPEC, EIEC, or STEC pathotypes should be confirmed by

biochemical assays and tested for the presence of virulence factors, and those screened as negative by the slide agglutination test should be tested for all categories using other methods, such as DNA-based assays (Piazza et al. 2010).

Early diagnostic procedures for EPEC consisted of metabolic characterization and serotype determination of 5–10 *E. coli* colonies isolated from stool culture (Alvarez et al. 1974; Toledo et al. 1983). Nowadays, EPEC bacteria are defined and differentiated through their capacity to produce localized adherence (LA) and attaching-effacing (A/E) lesions in tissue culture cells. Diagnosis of EPEC is now based on pathogenic characteristics that distinguish it from other *E. coli* pathotypes and subdivide EPEC into typical and atypical (which is presently the most frequent variant associated with acute and persistent diarrhea in children and adults and also with foodborne outbreaks). The PCR tests used reveal the presence or absence of genetic elements such as *eae*, *bfp*, and *stx*, which code for intimin, bundle-forming pili, and Shiga toxins, respectively.

ETEC bacteria were originally presumed to be more widely prevalent than EPEC. Their detection was initially performed using supernatants obtained from single *E. coli* colonies and by laborious procedures such as rabbit ileal loop test, suckling mouse assay, or cytopathic effect studies on Vero, CHO, or Y1 adrenal cell monolayers. ELISA assays were then developed using the GM_1 receptor to bind heat-labile toxin obtained from filtered culture supernatants or employing a competitive test for heat-stable toxin, which replaced former procedures (Torres et al. 2001; Bläck et al. 1979; Rivas et al. 1989). The pooling method (Rivas et al. 1989) was regionally used to recognize usually low proportions of positive colonies in ETEC-infected stool cultures. Positive samples gave way to single toxigenic culture analysis derived from individual colonies, which were also later serotyped and examined by dot blot immunoassay technique used to detect colonization factors (CFs). Actually, this pathotype was not usually found as the most frequent in surveillance studies or associated with diarrhea during outbreaks in the Americas. However, ST-positive strains have been associated with diarrheal disease, and ETEC is perennially considered the most common cause of "travelers' diarrhea" (Reis et al. 1982; Torres et al. 2001; Fleckenstein et al. 2010). Besides LT and ST detection, identification of ETEC strains should include complementary PCR assays for the detection of virulence genes such as *clyA*, *eatA*, *tia*, *tibC*, *leoA*, and *east-1* (Fleckenstein et al. 2010).

EIEC was initially diagnosed in stool cultures by selecting lactose-positive or -negative *E. coli* strains, which are usually nonmotile and lysine decarboxylase negative, assuming that invasive properties resembling those of *Shigella* were accompanied by similar metabolic pathways (Toledo and Trabulsi 1983; Chinen et al. 1993). Invasive strains were linked to certain serogroups, so efforts were required, like EPEC studies, to prepare, titrate, and use polyvalent and monovalent EIEC rabbit antisera for slide and tube agglutination reactions. Guinea pig Sereny eye test and tissue culture assays were more markedly limited to reference laboratories. EIEC invasive capacity can be evaluated using HEp-2 cell cultures. A useful test was also performed by analyzing suspect colonies through an in house virulence marker antigen (VMA) ELISA, using a rabbit antiserum prepared with an invasive

strain carrying VMA; the serum was absorbed with an attenuated, noninvasive variant of the same strain, obtained by successive subcultures in laboratory media (Pál et al. 1985). Further evidence, along with our own experience, has shown that biochemical properties and antigenic characteristics of strains pertaining to this pathotype are not restricted to original definitions, and include lysine-positive or indole-negative, motile strains of a variety of serogroups and serotypes. At present, initial screening through amplification of *ipaH* or other DNA sequences such as the invasion-associated locus gene (*ial*) is required (Escher et al. 2014).

EAEC is a very heterogeneous group, where it is the most difficult to categorize. The defining feature of EAEC is its characteristic aggregative adherence (AA) pattern to human epithelial cells on a glass substrate and to each other in a distinctive stacked brick formation. Thus, the gold standard method to identify EAEC is to culture five colonies per patient in static Luria-broth at 37 °C and then to infect semiconfluent HEp-2 cells for 3 h and look for the typical adherence pattern (Cravioto et al. 1991; Rüttler et al. 2006). However, this test requires specialized facilities and is time consuming to set up, and besides, it is very prone to contamination, restricting its use only to reference laboratories. Considering these difficulties, DNA probes were included as a valuable tool for EAEC detection (Levine et al. 1988; Regua-Mangia et al. 2004).

Initial screening can be done by amplification of both *aatA* and *aaiC* genes, but also testing for lysine decarboxylase activity and other phenotypic properties and employing bioassays. However, a multiplex PCR based on two genes encoded in the plasmid and two chromosome-borne genes is recommended to increase the ability to detect both typical and atypical EAEC strains. The *aggR*, *aatA* (Cerna et al. 2003; Jenkins et al. 2006), *aaiA*, and *aaiG* (Dudley et al. 2006; Morin et al. 2013) genes incorporated in the assay detecting *aaiA*, *aaiG*, *aggR*, and *aatA* demonstrated 94.8 % sensitivity and 94.3 % specificity and was able to efficiently detect both groups of EAEC among *E. coli* isolated from stool cultures (Andrade et al. 2014). This method should improve EAEC detection, since this pathotype is responsible for acute and persistent diarrhea in children and adults and is also associated with foodborne diarrheal outbreaks.

STEC group was almost directly included in these last diagnostic approaches after its recognition as an important cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Initial detection of Shiga toxin-producing strains and their infections was done through evaluation of cytotoxicity of culture or stool filtrates in eukaryotic cells (Konowalchuk et al. 1977; Karmali et al. 1983) and by stool culture and identification of certain serogroups (O26 and O157, respectively). However, DNA probes and later PCR techniques were soon found to be valuable tools for diagnosis (Pollard et al. 1990). Because sensitivity was much lower when the DNA template was prepared by direct extraction from feces, amplification techniques were initially limited to the cultured bacteria; the detection of *stx* genes was dependent on viable microorganisms and successful recovery from patients that had been improperly treated with antibiotics (Brian et al. 1992). Enrichment cultures are usually required due to these management difficulties and to the low proportion of positives for STEC bacteria in feces. Besides molecular tests, several commercially

available immunoassays can be employed for diagnosis, such as VTEC-Screen (Seiken, Japan), Premier EHEC (Meridian Bioscience, US), Ridascreen Verotoxin (R-Biopharm, Germany), ProSpecT Shiga Toxin (Alexon-Trend, US), Immunocard STAT! EHEC (Meridian Diagnostics, US), and Duopath Verotoxins Gold Labeled Immunosorbent Assay (Merck, Germany). Some of them differentiate between Stx1 and Stx2 while others do not. The reported sensitivities and specificities of these immunoassays vary according to test format and manufacturer. The standard by which each manufacturer evaluates its tests also varies; therefore, a direct comparison of performance characteristics of various immunoassays has not been performed (CDC 2009; Rocha et al. 2012).

2 Recent Advances in *Escherichia coli* Diagnosis Research: New Emerging Technology

The low level of most pathogens in fecal specimens requires specific and sensitive detection platforms, with new research aiming at techniques with high sensitivity and specificity, fast response time, accuracy, and affordable costs. The combined efforts of investigators and the availability of increasingly sensitive technologies are improving the quality and adding value to the services provided by clinical laboratories. The challenge surrounding a technologist's education is to learn new skills while maintaining expertise in classic techniques. Therefore, a combination of new technologies and classic techniques is central to success, combined with precise identification of all microorganisms encountered in the laboratory.

It is essential to understand that an early and accurate diagnosis achieved by laboratory technologists can have a rapid and significant impact on patient care by providing timely treatment, which may limit infection spread and disease extent. Likewise, a better patient management can reduce associated sequelae, unnecessary hospitalization, and inappropriate antimicrobial use with the associated reduction in costs (Buchan and Ledeboer 2014). In particular, DEC detection requires specialized tests, since they are indistinguishable from commensal *E. coli* strains based on biochemical tests. In addition, certain difficulties are observed for EIEC-*Shigella* differentiation, as both show similar biochemical and genetic traits.

2.1 Molecular Methods

Molecular methods, including PCR, microarray, and nucleic acid sequencing, have taken a prominent place in the clinical laboratory. These methods allow for sensitive and specific identification of microorganisms or genetic polymorphisms through amplification and detection of specific nucleic acid targets. To maximize the molecular testing benefits, diagnostics developers have begun to focus on technologies that employ both simplified technology and specimen preparation. With the

potential to further reduce turnaround time, which may positively impact patient care and reduce overall health care cost. They are also useful for detecting viable-but-nonculturable (VBNC) cells that may contribute to the spread of certain pathotypes, such as STEC (Moyne et al. 2013).

While the basic principle of nucleic acid amplification has not changed, technologies including amplification strategy, amplicon detection, multiplexing reactions, and entire process automation into sample-to-result platforms have provided several options from which the molecular microbiology laboratory can choose. One change in amplification strategy is the departure from PCR-based amplification to a technique termed loop-mediated isothermal amplification (LAMP), which is a simple, rapid, specific, and cost-effective nucleic acid amplification method. The assay is performed under isothermal conditions (constant temperature) and utilizes a strand displacement reaction, omitting the need for fluctuating temperatures to denature and to anneal DNA strands. Moreover, the LAMP assay displays very high specificity due to the use of four primers, which recognize six distinct target gene regions. Furthermore, the whole process from sample collection to detection requires only 90 min, and endpoint results can be achieved based on simple fluorescence and turbidity visualization. LAMP assays can be performed with simple equipment (e.g., water baths or controlled heating blocks), and the results can be determined by white precipitate direct visualization following centrifugation or changes in color upon addition of SYBR Green.

Liew et al. (2014) developed a novel LAMP assay targeting the invasion plasmid antigen H (*ipaH*) gene to rapidly detect the EIEC-*Shigella* group. On the other hand, Ravan et al. (2015) developed LAMP for the detection of *E. coli* O157:H7. Both LAMP assays proved to have a good sensitivity, when DNA extracts obtained from artificially contaminated food broths were tested. This approach could be extended to stool culture analysis. A major LAMP limitation is the inability to do multiplex assays. This is due to the nonspecific and indirect turbidity-based detection of the amplicon.

Enteric infections may exhibit nonspecific symptoms, which could be attributed to a number of different pathogens differing in genus, species, and serotype. Thus, it is very useful to implement multiplex PCR application with multiple primer sets into a single PCR assay for simultaneous detection of several targets. In this regard, multiplex systems such as end-time PCR and real-time PCR, varying the target or sample numbers to be processed, in different platforms, open and closed, and some automated, have been developed.

Regarding end-time PCR systems, two approaches may be mentioned that identify different DEC categories. Oh et al. (2014) proposed a one-step multiplex PCR assay using nine primer pairs to amplify nine virulence genes specific to the different *E. coli* pathotypes (stx_1 and stx_2 for EHEC; *lt*, *sth*, and *stp* for ETEC; *eaeA* and *bfpA* for EPEC; *aggR* for EAEC; and *ipaH* for EIEC). Also, Fialho et al. (2013) developed a two-system multiplex PCR for DEC detection. The multiplex-PCR system 1 contains primers for STEC (stx_1, stx_2), EPEC (*eae*, *bfpA*), aEPEC (*eae*), ETEC (*lt*, *st*), and EIEC (*ial*). System 2 contains primers for EIEC (*ipaH*) and EAEC (pCVD432) and includes primers for DA (*daaE*). Likewise, multiplex PCR is recognized as a very useful technique for molecular O antigen detection.

Based on O-antigen biosynthesis gene cluster analysis of all known *E. coli* O serogroups, 162 PCR primer pairs for the identification or classification of O serogroups were proposed by Iguchi et al. (2015). Of these, 147 pairs were used to identify 147 individual O serogroups with unique O-antigen biosynthesis genes, and the other 15 pairs were used to identify 15 groups of strains (Gp1–Gp15). Each of these groups consisted of strains with identical or very similar O-antigen biosynthesis genes, and the groups represented a total of 35 individual O serogroups. Subsequently, 20 multiplex PCR sets with 162 primer pairs were created. Sánchez et al. (2015) proposed a molecular approach for identifying different STEC serotypes. To develop a less laborious PCR method, the 21 serogroup-specific primer pairs were combined in three multiplex PCR assays aiming to detect the most clinically relevant STEC serogroups: Multiplex 1 (O5; O91; O26; O103; O145; O121; and O111), Multiplex 2 (O55; O128; O113, O146; O76; O45; and O177), and Multiplex 3 (O157; O15; O104; O118; O123; O165; and O172). The molecular serotyping methods were shown to be a faster, simpler, and less expensive technique than traditional serotyping, also enabling the detection of *E. coli* O antigen sequences, even when the bacteria cannot express them. As a consequence, the PCR assays could be an efficient and convenient strategy for strain serotyping in microbiology laboratories, especially in those where PCR is already a routine tool, and thus, their development could benefit clinical diagnosis. End-point multiplex PCR is one of the most useful diagnostic methods; however, this method requires gel electrophoresis analysis, which is labor intensive and can easily result in carryover contamination.

Real-time PCR in both approaches, the rtPCR SYBR green melting curve and PCR-probe based tests, are the options created for overcoming limitations, because amplification and detection are performed at the same time. Qin et al. (2015) used rtPCR SYBR green melting curve to detect STEC strains stx_1, stx_2, and $rfbE_{O157}$ virulence genes, and the results could be obtained from the melting peak. This PCR assay using enriched stool specimens demonstrated greatly improved sensitivity for STEC detection, especially in pediatric settings, where rapid sensitive diagnosis can prompt specific clinical actions. On the other hand, Youmans et al. (2014) used this approach not only to identify ETEC strain genes (*eltA*, sta_1, and sta_2) but also to quantify them in clinical stool samples. Reliable quantification of ETEC toxin genes is an important feature of this qPCR assay. A quantitative assay used for toxin quantification could improve diagnosis and potentially prevent unnecessary treatment regimens. High-resolution melting analysis (HRM) is very sensitive, but special attention should be paid to a number of factors affecting the PCR setup. With PCR reactions having similar efficiencies, the fluorescent level reached at the end point will be most similar, resulting in optimal HRM (Kagkli et al. 2012).

Hu et al. (2014) explored a probe-labeling strategy that significantly increased the target volume of real-time PCR in one reaction. The labeling technique, called Multicolor Combinatorial Probe Coding (MCPC), uses a limited number (n) of different fluorophores in various colors and combinations to label each probe, where $2^n - 1$ genetic targets can be detected in one reaction. In addition, MCPC was improved from a one-primer pair setting to a multiple-primer pair format, through the Homo-Tag Assisted Non-Dimer system, allowing multiple primer pairs to be

included in one reaction. By using a new design of modified MCPC molecular beacon and primers with homo tail sequences, a two-tube multiplex rtPCR for the identification of five *E. coli* pathotypes, including ETEC, EAEC, EPEC, EHEC, and EIEC, was developed and optimized by Chen et al. (2014). They established a two-tube multiplex real-time PCR assay for simultaneously detecting in tube one (*stp*, *sth*, *lt*, *aggR*) and in tube two (stx_1, stx_2, *eaeA*, *escV*, *ipaH*) genes with different probes and primer pairs for each one, but with the same thermocycling conditions.

The TaqMan Array Card (TAC) system is another strategy to overcome multiplexing capacity limited by the availability of fluorescent dyes and platforms. It is a 384-well spatial singleplex real-time PCR format that has been used to detect multiple infection targets. Liu et al. (2012) used this format to develop an enteric TAC to detect 19 enteropathogens, including viruses (adenovirus, astrovirus, norovirus GII, rotavirus, and sapovirus), bacteria (*Campylobacter jejuni*/*C. coli*, *Clostridium difficile*, *Salmonella*, *Vibrio cholerae*, DEC strains including EAEC, ETEC, EPEC, and STEC, *Shigella*/EIEC), protozoa (*Cryptosporidium*, *Giardia lamblia*, and *Entamoeba histolytica*), and helminths (*Ascaris lumbricoides* and *Trichuris trichiura*).

Primers and probes were newly designed or adapted from published sources and spotted onto microfluidic cards. This format is open modular, so once the assay performance is established under universal cycling conditions, different pathogen combinations can be spotted for the specific purpose of the end user. Investigators have emphasized that the cost per sample and turnaround time of TAC are also significantly less than that of conventional methods for each pathogen. However, this approach is designed for analysis of eight samples per plate, which requires extraction and manual setup of multiple real-time PCR wells per specimen. For laboratories with larger specimen volumes or limited staffing, the manual processing setup of reactions can complicate assay setup, and the handling of strains from similar origins may also be a potential source of cross-contamination of specimens.

The Prodesse® ProGastro SSCS Assay (Gen-Probe Prodesse) is a commercially available multiplex real-time PCR assay composed of seven TaqMan probes and different primer sets divided into two master premixes; the SSC master mix detects and differentiates *Salmonella*, *Shigella*, and *Campylobacter* species (but not *C. jejuni* and *C. coli*), and the S (STEC) master mix detects and differentiates the Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) genes as an indicator of Shiga toxin-producing *E. coli*. The ProGastro SSCS assay is highly sensitive and specific in a clinical setting when compared with culture for the identification of *Campylobacter* spp. (*C. jejuni* and *C. coli*), *Salmonella* spp., and *Shigella* spp. or with broth enrichment followed by EIA for the identification of STEC isolates in stool specimens (Buchan et al. 2013). Drawbacks of the assay are the requirement for prior offline extraction of nucleic acids from stool specimens and the need to set up duplicate real-time PCRs so that all targets are detected.

The BD Max enteric bacterial panel (BD Diagnostics) is another option of TaqMan multiplex nucleic acid amplification assay designed for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. (*C. jejuni* and *C. coli*), and Shiga toxin genes (stx_1/stx_2) in stool specimens. The BD Max system is a walk-away PCR instrument that can process the specimen and amplify and detect nucleic acids

in a batch of up to 24 samples in 3 h using only 1.25 min of operator time. The throughput and ease of use of this methodology may provide advantages to many laboratories, improving the detection of bacterial stool pathogens and the speed of reporting results.

Like in other multiplex PCR assays, this test is intended for use in conjunction with clinical presentation, laboratory findings, and epidemiological information, as an aid in the differential diagnosis of *Salmonella*, *Shigella*/EIEC, *Campylobacter*, and STEC infections. In a large multicenter study, the BD Max EBP assay showed superior sensitivity compared to conventional methods and excellent specificity for the detection of some enteric bacterial pathogens in stool specimens (Harrington et al. 2015). These test results should not be used as the sole basis for diagnosis, treatment, or other patient management decisions. Positive results do not rule out coinfection with different DEC pathotypes or other organisms that are not detected by this test and may not define the sole source or definitive cause of a patient's illness. Negative results in the setting of a clinical illness compatible with gastroenteritis may be due to infection caused by pathogens that are not detected by this test.

In contrast, the commercially available version of a closed system multiplex PCR, the FilmArray GI Panel (BioFire Diagnostics), allows simultaneous detection of 22 common diarrheal agents, including bacteria, viruses, and protozoa as follows: bacteria (*C. jejuni*, *C. coli*, *C. upsaliensis*, toxigenic *C. difficile*, *Plesiomonas shigelloides*, *Salmonella*, *Vibrio parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *Yersinia enterocolitica*, ETEC (*lt*/*st*), EPEC, STEC (stx_1/stx_2), including *E. coli* O157, *Shigella*/EIEC, EAEC, viruses (adenovirus F40/41, astrovirus, norovirus GI/GII, rotavirus A, and sapovirus "genogroups I, II, IV, and V"); and protozoa (*Cryptosporidium*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, and *Giardia lamblia*).

The FilmArray system is a sample-to-result multiplex PCR contained within a single test pouch. However, FilmArray analyzes one sample at a time and therefore is best suited for laboratories requiring a low throughput, where its use may be best suited to critically ill or immunocompromised patients rather than for routine testing. The ability to cover a broad spectrum of gastrointestinal pathogens in a single test is a multiplex technology advantage. Nevertheless, the impact of multiplex tests on patients with gastrointestinal illness management and treatment is still unclear, since no standard therapy exists for some of the pathogens represented in these panels. Furthermore, a lot of possible pathogen combinations can be detected by this panel, and the significance of detected coinfections may be difficult to understand because the clinical implications of specific pathogen combinations are not well documented or understood. Therefore, rapidly determining which potential pathogens a patient harbors becomes a challenge when the results are obtained with such methods. This is an important step in formulating an effective treatment plan and applying appropriate infection control measures (Khare et al. 2014).

Several approaches referred to as microarrays have been explored to expand the number of detectable targets in a single multiplex nucleic acid test. Liquid-array technology known as LuminexTag gastrointestinal pathogen panel (GPP) (Luminex Corporation) involves an initial multiplexed PCR step, followed by a target-specific primer extension that incorporates a unique nucleic acid "tag" and biotin label into

each target amplicon. Tagged amplicons are then incubated with various fluorescent microbeads, each type coated with a unique antitag sequence. Amplified target sequences with incorporated tags complementary to those on a specific bead will hybridize. Finally, a streptavidin–fluorophore conjugate is added and hybridized to biotin-labeled amplicons immobilized on the beads. Target detection is carried out using two lasers that examine each bead for the presence of captured amplicon as indicated by the streptavidin–fluorophore conjugate and the identity of the amplicon is indicated by fluorescence of the specific bead for each antitag. The xTAG test for gastroenteritis agents (xTAG GPP) includes: adenovirus subtypes 40/41, norovirus genogroups (GI/GII), group A rotavirus, *Campylobacter* spp., *C. difficile* toxin A/B, *E. coli* O157, ETEC LT/ST, *Salmonella* spp., STEC stx_1/stx_2, *Shigella*/EIEC spp., *V. cholerae*, *Y. enterocolitica*, *Cryptosporidium hominis*, *E. histolytica*, and *Giardia duodenalis*. EPEC and EAEC are not detected.

It is important to keep in mind that at first, an external nucleic acid extraction is needed. After extraction and purification, a multiplex reverse transcription PCR and hybridization with xTAG beads are performed, using a conventional thermocycler. Following hybridization, fluorescence is generated and recorded for each xTAG bead population by the Luminex 100/200 instrument. The data are analyzed using the xTAG Data Analysis Software for GPP. The Luminex platform is an open system, it requires 60 min of hands-on time, and has a turnaround time of 5–6 h. Despite the long turnaround time, the Luminex system processes up to 96 samples, making it suitable for high-volume reference laboratories (Khare et al. 2014). This approach was used to investigate the enteric pathogens infecting people living in Côte d'Ivoire, in sub-Saharan Africa. The diarrheal disease surveillance showed evidence that xTAG GPP improved the available knowledge about the etiology of symptomatic and asymptomatic enteric mono- and coinfections in Côte d'Ivoire (Di Cristanziano et al. 2015).

The GoldenGate assay is a new emerging molecular technology developed by Illumina (Illumina) and adapted for enteropathogens detection (Lindsay et al. 2013). The GoldenGate assay protocol consists of five phases: activation, extension/ligation, PCR, hybridization, and imaging. The GoldenGate assay be composed of allele-specific oligonucleotides (ASOs) and locus-specific oligonucleotides (LSO) that are hybridized to the target sequence and then ligated together. The products are PCR amplified using biotinylated type (TBP) and fluorophore type (TFP) primers. The primers used by Lindsay et al. (2013) were designed using GoldenGate software to identify EAEC, ETEC, EPEC, *Shigella* spp., *Campylobacter jejuni*, *S. enterica*, and *Aeromonas* spp.

2.2 *Recent Advances in Sequencing Technologies*

In the last decade, next-generation sequencing (NGS) appeared to be replacing Sanger's method. Genome sequencing is becoming increasingly rapid and less expensive (Bertelli and Greub 2013; ECDC 2015). Bacterial genomics research has

been used to study molecular pathogenesis (genetic basis of virulence traits, host–pathogen interactions, antibiotic resistance), phylogenetic analysis (population genomics, strain emergence, outbreak investigation, pathogen discovery), and for clinical tool development (vaccines, diagnostics, and therapies have been identified). The recent tremendous advances in benchtop NGS instrumentation (454 GS Junior, Ion PGM and MiSeq), laboratory automation, and bioinformatics tools have given all microbiology laboratories access to microbial whole-genome sequencing (WGS), which is becoming widely adopted as a routine tool by clinical microbiology laboratories (Bertelli and Greub 2013).

The identification of pathogenic *E. coli* associated with diarrheal diseases has always been complex to perform in microbiology laboratories, and in many cases, it is done just at reference laboratories. Molecular techniques, included in most workflow charts, have been useful tools that help in the diagnosis and pathogen characterization. In the last years, WGS was used in different research investigations in diarrheagenic *E. coli* diagnosis, characterization, and subtyping. As WGS became less expensive, it was adopted as a useful tool for fast and accurate pathogen identification and typing, and it is suggested to be applied in surveillance and outbreak detection as part of public health strategies. Different WGS approaches have been used for detection of virulence genes, essential to differentiate the strains in the distinct diarrheagenic *E. coli* categories (Ashton et al. 2015; Pattabiraman and Bopp 2015; Hayashimoto et al. 2015).

In 2014, a real-time evaluation of WGS for routine typing and surveillance of STEC was published by Joensen et al. The bioinformatics analysis for species determination, multilocus sequence typing (MLST), phylogenetic relationship determination, and even a specific detection of *E. coli* virulence genes using the free web tools at the Center for Genomic Epidemiology (CGE; www.genomicepidemiology.org) were described. It was also demonstrated to be a robust method for assigning Stx cytotoxin (*stx*) subtypes, and for real-time clustering of isolates in agreement with the epidemiological profile, enabling discrimination between sporadic and outbreak isolates (Joensen et al. 2014).

The replacement of traditional serology-based *E. coli* typing with WGS at national reference laboratories is supported by the above-mentioned user-friendly, freely available data analysis web tools (CGE) (Joensen et al. 2014). It was demonstrated that WGS-based in silico serotyping for *E. coli*, using CGE tools, is applicable for routine typing and surveillance. The use of CGE SerotypeFinder was evaluated on 682 *E. coli* genomes. In total, 601 and 509 isolates were included for O and H typing, respectively. SerotypeFinder for WGS-based O (O-antigen genes *wzx*, *wzy*, *wzm*, and *wzt*) and H (flagellin genes *fliC*, *flkA*, *fllA*, *flmA*, and *flnA*) typing predicted 560 of 569 O types and 504 of 508 H types, consistent with conventional serotyping (Joensen et al. 2015).

WGS is now currently used for supporting outbreak investigation, identifying the associated pathogens. Jenkins et al. (2015) described a public health investigation of two simultaneous outbreaks of STEC O157 PT2 that were both linked to watercress consumption but were associated with different contamination sources. Other O157 outbreaks in England and Wales were detected using WGS instead of

traditional typing (Dallman et al. 2015). These clusters were previously unidentified, often widely geographically distributed and small in size. Phylogenetic analysis by WGS facilitated identification of temporally different cases sharing common exposures, delineating those that shared epidemiological and temporal links. WGS allows timely resolution of outbreak clustering with unprecedented specificity and sensitivity, which will facilitate targeted and appropriate public health investigations. The first time that WGS was applied, demonstrating to be a powerful tool for taking rapid public health actions, was in the multidrug-resistant EAEC/EHEC O104:H4 outbreak that occurred in Germany in 2011 (Mellmann et al. 2011). Even now, it is also useful in following up the strains through the years. Ferdous et al. (2015) described the circulation, during 2013, of EAEC Stx2a+ O104:H4 strains highly similar in their core genome to the German 2011 outbreak clone. WGS not only provided a detailed isolate characterization, but also enabled the differentiation of the 2013 isolates from the 2009 and 2011 isolates, with high discriminatory power, expediting the use of WGS in public health services to rapidly apply proper infection control strategies. Other STEC/ETEC hybrid strains, possessing both *stx* and *est* genes, were sequenced using PacBio RS sequencer. This study showed that different pathotype-associated *E. coli* virulence genes could coexist in strains originating from different phylogenetic lineages (Nyholm et al. 2015).

2.3 Mass Spectrometry Methods

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) has been increasingly used in recent years for the rapid and reliable identification of microorganisms. This method allows the identification of unknown bacteria within a few minutes without special techniques or use of costly reagents. Samples are prepared by mixing the analyte with a matrix made of small acid molecules that exhibit a strong absorption in the wavelength range used by the laser device (DHB 2, 5-dihydroxybenzoic acid and CHCA α-cyano-4-hydroxycinnamic acid). The sample crystals with acid matrix then absorb energy from the laser, leading to desorption and analyte ionization in the gas phase. Ions are further accelerated through an electrostatic field into the high vacuum flight tube until they reach the detector, with smaller ions traveling faster than larger ones. Thus, the time of flight (TOF) required to reach the detector is dependent on the mass and bio-analyte charge, resulting in a unique spectral profile unique for a given species, composed of peaks ranging usually from 2 to 20 kDa (De Carolis et al. 2014).

The conventional determinations are based on fingerprinting analysis, to compare MS patterns of unknown samples with reference spectral collections in accumulated databases. MALDI-TOF-MS analysis has great potential as an advanced identification method for microorganisms in routine laboratory settings because of the significantly reduced times required as compared to molecular biological methods.

To date, accurate identification has depended greatly on the number of database entries; a reference database update is needed to improve the identification performance of MALDI-TOF.

Numerous studies using MALDI-TOF-MS have proven that SARAMIS™ (BioMérieux) or Biotyper (BrukerDaltonics) fingerprinting databases are useful for bacterial identification but only at the genus level. Serotype- or species-level identification is not always successful because slight mass-to-charge ratio [m/z] differences and individual MS peaks are not taken into account in conventional finger printing analyzing software. *Shigella spp*. is so closely related to *E. coli* that routine MALDI-TOF-MS cannot reliably differentiate them. Biochemical and serological methods are typically used to distinguish these species; however, "inactive" *E. coli* isolates are biochemically very similar to *Shigella* and thus pose a greater diagnostic challenge.

ClinProTools (BrukerDaltonics) software was proposed by Prasanna and Fisher (2013) to discover new potential MALDI-TOF-MS biomarker peaks and to generate classification models, on the basis of a genetic algorithm to differentiate between *Shigella* spp. and *E. coli*. This assay could be adopted by clinical laboratories to rapidly distinguish inactive and other non-lactose-fermenting *E. coli* isolates from *Shigella*, although it requires large cohorts of *E. coli* and *Shigella* isolates to generate a larger database. The ability to transfer MALDI-TOF-MS spectra between laboratories may promote the use of wider data analysis approaches.

A strategy to discriminate O26, O111, and O157 *E. coli* serotypes from others, using MALDI-TOF-MS, is based on the S10-*spc*-alpha operon gene-encoded ribosomal protein mass spectrum (S10-GERMS) method (Ojima-Kato et al. 2014). When using MALDI-TOF-MS data to identify microorganisms, most high-intensity MS peaks detected are derived from ribosomal proteins encoded in the S10-*spc*-alpha operon. The S10-GERMS method provides theoretical values [m/z] based on gene sequences in the S10-*spc*-alpha operon; therefore, "proteo-typing" based on m/z biomarker peaks can be used as an accurate approach. The new strategy includes the following steps: recording the MS m/z specific peaks of biomarkers generated using the S10-GERMS method; importation of data matrices of the m/z peaks and intensities from isolated microorganisms obtained by MALDI-TOF-MS; and finally, matching of recorded MS peaks with those of analyzed microorganisms using "Strain Solution" software. The study results suggested that MALDI-TOF-MS analysis with Strain Solution could be effectively applied to discriminate *E. coli* serotypes O157, O26, and O111. Given its great versatility, the use of this method for species- or serotype-level identification is recommended.

On the other hand, Tagg et al. (2015) described the development of a MALDI-TOF-MS method validation for multilocus sequence typing (MLST) of *E. coli*. Effective infection control and management requires reliable and robust subtyping schemes. Thus, the molecular method in conjunction with the MALDI-TOF-MS contributes to improving their particular properties. The high automation level ensures reproducibility, minimizes costs, and reduces the need for specialized technical expertise. The rapid availability of MLST MALDI-TOF-MS results could help

in the application of quick short-term infection control strategies, and over time, it would enable the accumulation of valuable epidemiological information. Both of these features are important for improving public health outcomes.

The Ibis T5000 Universal Biosensor is a pathogen detection platform capable of broad range pathogen identification and strain typing. This method uses an automated process to aliquoted extracted DNAs, amplifying genes using PCR primers for microorganism detection, and then subjecting the amplified products to electrospray ionization mass spectrometry (ESI-MS). The mass spectrometer measures the PCR products to calculate a molecular weight, and the nucleotide composition can be deduced for each amplicon present. The Universal Biosensor assay utilizes a known DNA sequence base composition database to determine which microorganisms are present (Ecker et al. 2008). Six primer pairs targeting 16S and 23S rRNA genes were used to identify microbes (Gram-positive, Gram-negative, aerobic, and anaerobic), and other primer pairs were used to characterize 17 additional genes (*ctxA*, *ctxB*, *east-1*, *east-2A*, *estA1*, *eltA*, *eltB*, *stx*$_1$*A*, *stx*$_1$*B*, *aggR*, *aatA*, *eae*, *invA*, *ipa*H, *ipaA*, *ipaC*, and *ipaB*) and to identify the pathogen-specific markers (Lindsay et al. 2013). Universal Biosensor assays could be used to test different groups of microorganisms, including *Campylobacter* spp., *Clostridium* spp., *E. coli*, nontyphoidal *Salmonella* spp., *Salmonella enterica* serovar Typhi, *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*, *V. cholerae*, *V. parahaemolyticus*, ETEC, EPEC, EAEC, *Yersinia spp*., and the genes ctxA, ctxB, eae, east-1, invA, ipaB, ipaC, ipaD, and ipaH.

Chromatographic retention time and MS fragmentation methods detecting Stx1 and Stx2 in bacterial media and human serum have been developed (Silva et al. 2015). A synthetic gene to produce a single protein (^{15}N-Stx-ISP) was used as internal standard. This method can be used to differentiate the known Stx2 and Stx1 subtypes in complex media. The entire procedure can be done in approximately 5 h. The serum sample is digested with trypsin and sequentially filtered through a 10,000- and 5000-Da MWCO filter. The internal standard is added and the sample is run on the instrument. The appropriate ^{15}N-labeled internal standard is used to identify the peak corresponding to analogous unlabeled peptides based on their identical physicochemical properties. The area ratio of the unlabeled peptide to that of the added internal standard may be calculated and used to quantitate the amount of Stx present in the sample.

2.4 Immunological Tests

Despite the current efforts to develop reliable and low-cost diagnostic assays, DEC detection assays are currently unavailable in most developing countries, except for identifying the STEC pathotype. Characteristic virulence gene identification by PCR is an obvious choice to determine one or multiple virulence factors. Nevertheless, gene detection does not indicate virulence factor expression/production, and furthermore, the great genetic diversity that the strains exhibit makes molecular detection

difficult, which is also dependent on the primer sequences used. Considering the expression of virulence factors, other methods such as animal assays or in vitro cell culture cells have more disadvantages than advantages, because these methods are time consuming, laborious, and cumbersome for routine diagnosis. Thus, immunoassays can be considered an alternative method for the detection of virulence factors, for which polyclonal and/or monoclonal antibodies are necessary.

Considering EPEC and EHEC diagnosis, one of the main and common virulence factors is intimin, a 94-kDa outer membrane protein that is an excellent target for detection. In this regard, the intimin recombinant antibody (scFv-intimin) was evaluated by Caravelli et al. (2013), using a sensitive and specific immunofluorescence assay. scFv-intimin proved to detect typical EPEC, atypical EPEC, and EHEC isolates. Immunofluorescence is an effective and rapid method and scFv-intimin is an excellent tool for diagnosis of diarrhea caused by EPEC and EHEC (Fig. 15.1a).

Rapid and low-cost diagnosis of EPEC/EHEC infections is extremely needed considering their global prevalence, the severity of associated diseases, and the fact that antibiotics used to treat EHEC infections can be harmful. For EHEC, the detection of Stx toxins has already been developed, but for EPEC, an internationally recognized standard diagnostic test is lacking. An interesting diagnostic tool for both pathogens, which can be employed in poorly equipped laboratories, has been proposed. It encompasses a rapid agglutination test using latex beads coated with anti-EspB mAb, standardized for sensitivity and specificity required for the diagnosis of enteropathogenic diseases (Rocha et al. 2014) (Fig. 15.1b).

Regarding STEC diagnosis, some commercial immunoassays are available, but most of them recommend culturing stool specimens before analysis. If culture were done before analysis, the real concentration of Stx in patient's stool could not be established. A quantitative bead sandwich enzyme-linked immunosorbent assay, without precultivation of stool specimens, was performed by Yamasaki et al. (2015). The Fab' of antitoxin IgG was conjugated with horseradish peroxidase, and tetramethylbenzidine was used as the substrate, combined with a 6.5 mm diameter polystyrene bead as the solid phase coated with the anti-toxin IgG. Although some inhibition was observed with control stool specimens, information about ranges of Stx concentration in a patient's stool was revealed in the bead-ELISA system.

Early and accurate diagnosis of STEC infections after diarrhea onset continues to challenge clinicians due to the small number of cases confirmed by bacterial isolation and/or toxin detection. Accordingly, alternative laboratory diagnostic methods have been used, such as those that detect antibodies against Shiga toxins or STEC antilipopolysaccharide (LPS). Anti-LPS antibodies combined with stool culture and free fecal Shiga toxin detection has improved diagnostic performance. For these reasons and since the humoral immune response to LPS is dominated by antibodies against the O-polysaccharide portion, Melli et al. (2015) exploited a bacterial glyco-engineering technology to develop recombinant glycoproteins consisting of the O157, O145, or O121 polysaccharide attached to a carrier protein, to use them as serogroup-specific antigens in indirect enzyme-linked immunosorbent assays (glyco-iELISAs). The antigen synthesis strategy is based on the *C. jejuni* N-glycosylation system that can be transferred to *E. coli* (Wacker et al. 2002). The

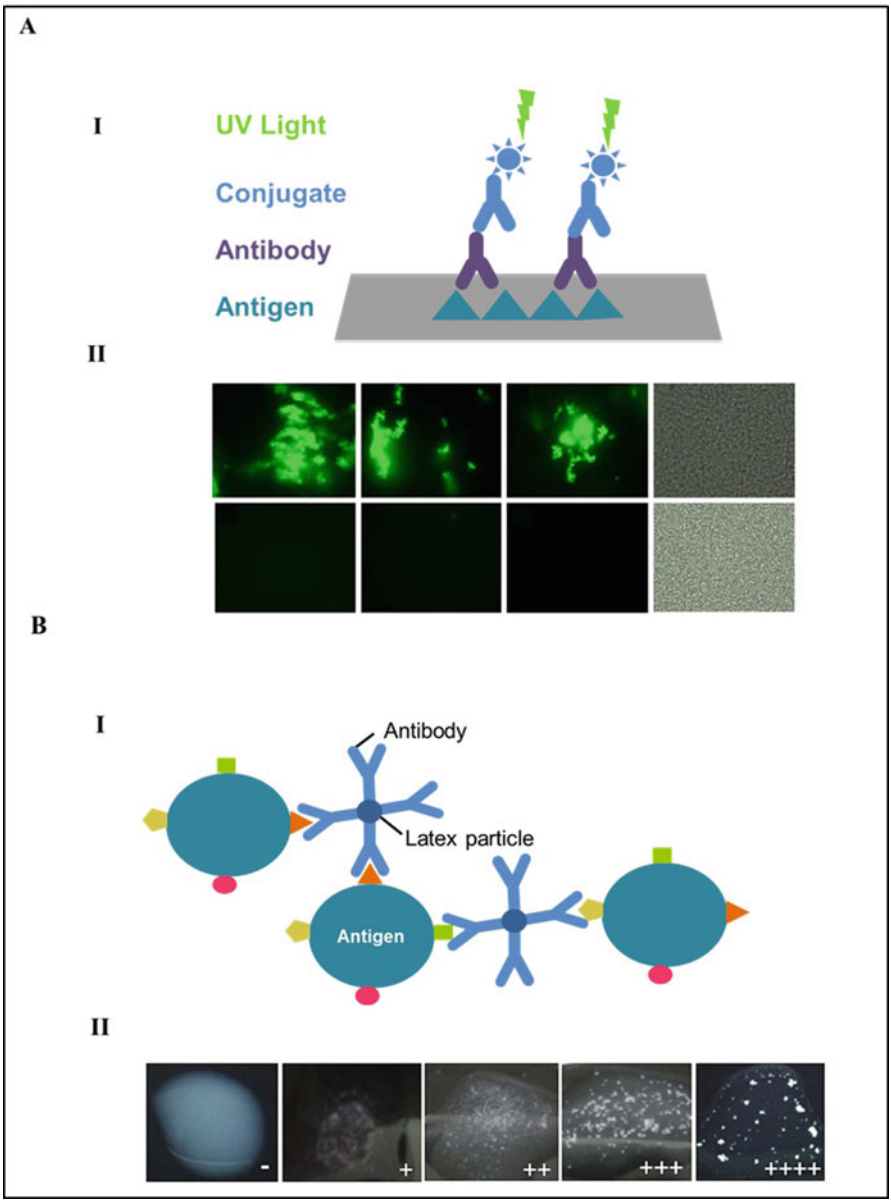

Fig. 15.1 Representation of immunodiagnostic tests. (**a**) Anti-intimin immunofluorescence test; (I) representative scheme of immunofluorescence steps; (II) reactivity of scFv-intimin in immunofluorescence assay against positive and negative isolates (Caravelli et al. 2013). (**b**) Rapid agglutination test using latex beads; (I) representative scheme of agglutination assay; (II) negative and semiquantitative positive (from + to ++++) agglutination pattern with anti-EspB mAb-coated beads (Rocha et al. 2014)

C. jejuni N-oligosaccharyltransferase (OTase) PglB can transfer an array of LPS O polysaccharides from lipid donor to carrier proteins in a system that combines both the LPS biosynthesis pathway of Gram-negative bacteria and the N-glycosylation pathway of C. jejuni. In this system, several glycosyltransferases add sequentially the sugars required for the synthesis of the O polysaccharide to the lipid carrier undecaprenol-phosphate (Und-P), on the cytoplasmic side of the membrane. Next, the lipid-linked oligosaccharide is flipped into the periplasm, polymerized, and transferred by PglB to a carrier protein (AcrA) instead of transferring it to the lipid A core of LPS. Therefore, the resulting O-polysaccharide-protein conjugates can be produced and purified from cultures of nonpathogenic bacteria. In this way, the recombinant glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA were produced by Melli et al. (2015) in *E. coli* coexpressing PglB, AcrA-His tag, and the cluster of genes required for the synthesis of the corresponding O polysaccharide.

The glyco-engineering method overcomes many of the inherent disadvantages of the traditional chemical methods used for the production of such glycoconjugates. With this technology, there is no need to purify the acceptor protein and LPS separately, and no chemical treatments are required for the isolation of the O polysaccharide from it. Additionally, no chemical cross-linking of the carbohydrate to the protein is required, which allows the production of the conjugates with a defined and reproducible sugar pattern, and this is important because the length of the O polysaccharide and the glycosylation process are controlled in vivo. Finally, due to the great versatility of this technology, it should be very simple to expand this antigen panel to detect other serogroups currently important for the disease in different parts of the world, such as O26, O103, O111, and O45, which are included as part of the "big six" non-O157 STEC serogroups (together with O145 and O121). This method could be adapted to quickly develop new antigens for emerging STEC serogroups (e.g., the O104:H4 outbreak that occurred in Germany), allowing a timely response in case of an outbreak and potentially improving control measures. The recombinant glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA are serogroup specific, and no cross-reactivity with other glycoproteins has been observed by either immunoblot or glyco-iELISAs. This serospecificity may be due to the fact that only the O-polysaccharide, the outer moiety of LPS, is coupled to the carrier protein. Therefore, glyco-based assays may be more specific than serological tests currently used, and due to their serospecificity, they may be valuable laboratory tools, not only for diagnosis, but also for studying the distribution of STEC serogroups for epidemiological purposes.

Interestingly, a specific IgM response was detected in almost all of the analyzed samples, indicating that it is possible to detect the infection even at the early stages of disease. More than 90 % of the samples obtained from HUS or bloody diarrhea (BD) patients, with confirmed STEC association, were serologically positive by the glyco-iELISAs. In all cases, the identified serotype was in accordance with the serotype of the isolated strain, demonstrating the value of these antigens, not only for the diagnosis of the infection, but also to quickly provide information about the serotype of the isolated strain. Additionally, samples obtained from patients with no confirmed STEC disease association were positive by the glyco-iELISAs, indicating

that these tests can improve disease diagnosis even in HUS patients for whom bacterial isolation and Shiga toxin detection have failed. This study indicates that glycoliELISAs, due to their serospecificities, are useful not only for infection detection but also for guiding the subsequent steps to confirm diagnosis.

3 *Escherichia coli* Diagnosis in America

The difference in DEC epidemiology on the American continent, from South to North, from West to East, determines also how the disease is diagnosed. In fact, considering developing countries, substantial reductions in deaths and complications due to diarrhea have been a result of improved sanitation and safe water, the promotion of breast-feeding and oral rehydration. However, despite these efforts, diarrhea continues to be an important cause of morbidity, because in some regions people do not have access to a sewage system (Navarro and Estrada-Garcia 2010; Vidal et al. 2010; http://portal.saude.gov.br; Lanata et al. 2013).

Except for Argentina, in which notification to the National Health Surveillance System is mandatory for cases of acute diarrhea (Rivas et al. 2010), in other Latin American countries, knowledge of epidemiology is based on published literature. The most prevalent DEC pathotypes are EAEC and EPEC, followed by ETEC and DAEC and in lower prevalence EIEC strains (Varela et al. 2007, 2015; Assis et al. 2014; Bueris et al. 2007; Moreno et al. 2010; Barletta et al. 2009; Ochoa et al. 2008, 2009, 2011; Lozer et al. 2013; Foster et al. 2015; Franzolin et al. 2005; Patzi-Vargas et al. 2015; Rúgeles et al. 2010; Hannaoui et al. 2010; O'Ryan et al. 2005; Riveros and Ochoa 2015; Gómez-Duarte et al. 2010; Pérez et al. 2010).

Human infections by STEC are endemic in Argentina, emerging in Chile and Mexico, and are mainly linked to O157 strains. In other Latin American countries, STEC (O157 and non-157) represents sporadic cases of diarrhea, bloody diarrhea, hemolytic anemia, and HUS (Rivas et al. 2010; Vidal et al. 2010; Guth et al. 2010; Varela et al. 2010; Navarro and Estrada-Garcia 2010).

Diarrhea caused by DEC (other than STEC) receives poor attention in industrialized American countries, such as USA and Canada. According to the Centers for Disease Control and Prevention (CDC), laboratory-confirmed foodborne bacterial infections in the USA were caused by *Salmonella spp.*, followed by *Campylobacter spp.*, *Shigella spp.*, STEC, and *Yersinia spp.* (CDC 2009).

Investigation of all DEC pathotypes is not routinely performed in American clinical laboratories through the described tests. It demands abundant resources, time, cumbersome laboratory work, and has limited impact on patients' management (except for those suspected of having STEC infections or foodborne outbreaks) (Nataro et al. 2006; Varela et al. 2015; Assis et al. 2014). Serotyping-based diagnosis is the only method available in settings of limited resources, utilizing either commercial or in-house antisera (Piazza et al. 2010).

In most countries in the Americas, detection and full characterization of DEC can only be performed in central reference laboratories. Surveillance studies of prevalent diarrheal agents, in particular DEC, are important to design specific con-

trol measures, vaccination strategies and treatment regimens (Kotloff et al. 2012), and to allow detailed strain analysis, which can provide important knowledge about host–microbe interactions and pathogenesis. They pave the way for the development and evaluation of new diagnostic tools and help in defining the role of DEC in certain diarrheal disease caused by pathotypes, such as DAEC or EAEC, which requires further analysis.

In areas where diarrheal diseases have the highest health impact, the materials and technical resources to carry out etiologic studies are extremely scarce or absent, especially in small laboratory facilities. This situation has also led some local services to establish collaborative links with international reference laboratories for developing surveillance studies or performing detailed analysis of DEC strains recovered in that region.

Multiplex reactions have been developed by several groups, allowing initial detection of one or more pathotypes in sweeps or colony pools from stool cultures, but at present, different DNA-based techniques are employed in few laboratory settings studying DEC infections (Nataro et al. 2006; Assis et al. 2014; Aranda et al. 2004; Vidal et al. 2005; Bueris et al. 2007; Barletta et al. 2009; Gómez-Duarte et al. 2009, 2010; Ochoa et al. 2011; Fialho et al. 2013; Lozer et al. 2013; Foster et al. 2015; Botkin et al. 2012).

In 2012, the Global Enteric Multicenter Study group (GEMS) recommended using a robust and inexpensive standard approach for enteric pathogen detection in developing countries, beginning with classic bacteriological methods for the isolation of putative bacterial pathogens from feces, followed by molecular and/or phenotypic characterization of the microorganisms (Panchalingam et al. 2012). In this approach, stool samples collected in a transport medium (e.g., Cary-Blair) are cultured on different solid media and enrichment broths. The method for bacteriological isolation comprises the use of several solid media (e.g., MacConkey [MAC] and MacConkey sorbitol [SMAC] agar) and at least one enrichment broth. Subsequently, several lactose-fermenting (or non-lactose-fermenting), sorbitol-fermenting (or sorbitol nonfermenting) bacterial colonies resembling *E. coli* are analyzed using identification tests, and the colonies confirmed as *E. coli* are studied by PCR for virulence genes that define the DEC pathotypes.

At present, the GEMS recommended strategy is used by laboratories in several Latin American countries for DEC surveillance studies, including Bolivia, Colombia, Peru, Argentina, Chile, Brazil, México, Venezuela, and Uruguay. Local modifications to the method have been introduced regarding type of enrichment broth, primary isolation medium (MAC, SMAC, eosin-methylene blue agar), DNA extraction procedure (boiling or commercial kits), number of colonies studied, PCR type, and target genes and primer sequences (Varela et al. 2015; Patzi-Vargas et al. 2015; Vidal et al. 2005; Gómez-Duarte et al. 2010; Ochoa et al. 2011; Lozer et al. 2013; Foster et al. 2015; Hannaoui et al. 2010; Silveyra et al. 2015). This approach requires a minimum of 20–30 isolated colonies per plate, a set of efficient primers, and flexible interpretation criteria. If less than 20 colonies are obtained, a sample of confluent growth zone of the culture plate must be used. In this case, confirmation of positive results could be more difficult, especially when more than one virulence trait is detected.

The CDC in the US recommends for investigation of STEC a similar strategy to the general procedure proposed by GEMS, when dealing with feces from community diarrheal cases. STEC detection is based on stool culture of *E. coli* O157 using SMAC plates, enrichment broths, and screening by a DNA-based assay or with a direct antigen identification method for Shiga toxins 1 and 2 from supernatants of broths, after 16–24 h of incubation (CDC 2009).

The methods used in USA and Canada for DEC investigations are similar to those used in Latin American countries (Nataro et al. 2006; Botkin et al. 2012). In the last reference report, five lactose-fermenting and two non-lactose-fermenting colonies from MAC plates are pooled and subjected to direct PCR for detection of *E. coli* virulence factors. STEC screening is performed by EIA, and positive broths are isolated on MacConkey sorbitol for PCR analysis of individual colonies.

In Canada, the Alberta Provincial Pediatric EnTeric Infection TEam (APPETITE) recommended in 2015 the use of xTAG GPP directly on stool samples or rectal swabs, to study the causes of diarrhea in children. The aims of the proposal were as follows: to improve health through enhancement of enteric pathogen identification and to develop economic models, incorporating pathogen burden and societal preferences, to inform enteric vaccine decision-makers (Freedman et al. 2015).

It is worth mentioning that the method of choice will depend on the facilities of each laboratory, taking into account the advantages of each method, the characteristics of the diarrhea studied, and the clinical symptoms of the patients. Despite many efforts in standardizing methods, in which the detection can be made directly with stool specimens, isolation and presumptive identification are still necessary. Isolation of the pathogen largely depends on the collection and a proper transport of the specimens. The collection should be performed preferably at onset of illness, and the stools should be cultured as soon as possible in the laboratory (Piazza et al. 2010).

New technologies and approaches are required to increase speed and reliability for the detection of DECs from stool samples; e.g., new enrichment broths and isolation media are also needed for recovery and full characterization of all DEC strains. Associated high costs can only be afforded by central laboratories in special circumstances and not as part of routine diagnosis. Combined work is mandatory, assigning sentinel, surveillance studies, and initial screening during outbreaks to first-level laboratory services (which should also process all HUS-suspected samples), and submitting inoculated media and samples to reference laboratories.

Laboratories serving the first level of health attention should be strengthened in their capacity of performing classic initial methods of stool microbiological analysis (sample collection, inoculation of primary enrichment, and isolation media), in their ability to carry out simple and quick screening tests, such as immunochromatographic assays (Fig. 15.2), not only for STEC but also for ETEC or EPEC, and also to perform other methods that could be developed in the future to cover prevalent pathotypes in different countries. For the moment, DNA-based studies should be performed in second or third level central laboratories. As a result of these limitations, progress is needed in the overall capacity of laboratories to detect and study DEC strains from clinical cases and other sources.

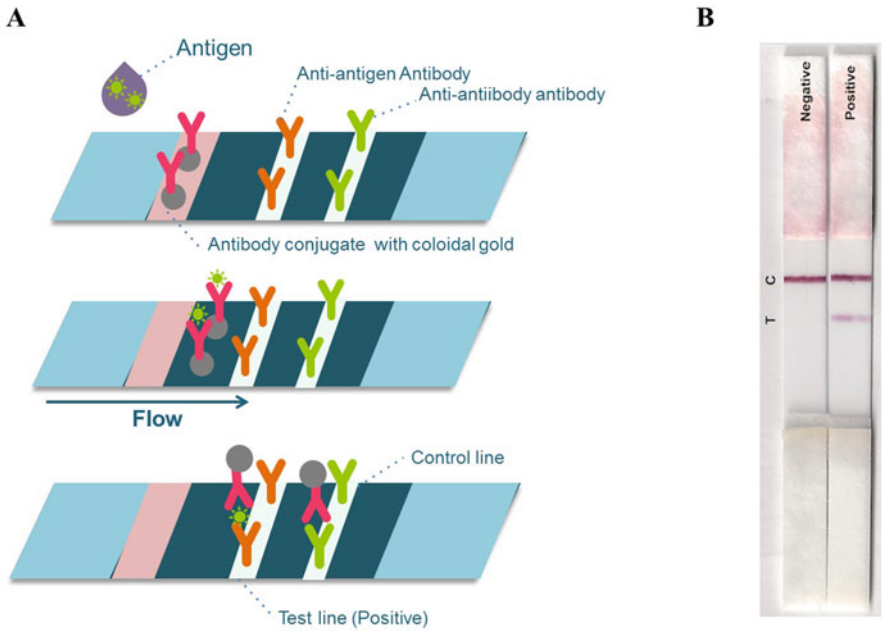

Fig. 15.2 Immunochromatographic test; (**a**) representative scheme of the immunochromatographic steps through the strip, which occur by capillarity; (**b**) Stx toxin detection by immunochromatographic assays, developed by Piazza's laboratory, showing the positive (presence of Stx) and negative controls (unpublished data)

References

Alvarez F, Hormaeche CE, Demarco R, Alía C, Schelotto F (1974) Consideraciones clínico-bacteriológicas de diarrea aguda de lactantes hospitalizados. Arch Pediatr Urug 45:210–221

Andrade FB, Gomes TAT, Elias WP (2014) A sensitive and specific molecular tool for detection of both typical and atypical enteroaggregative Escherichia coli. J Microbiol Methods 106:16–18

Aranda KRS, Fagundes-Neto U, Scaletsky ICA (2004) Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic Escherichia coli and Shigella. J Clin Microbiol 42:5849–5853

Ashton PM, Perry N, Ellis R, Petrovska L, Wain J, Grant KA, Jenkins C, Dallman TJ (2015) Insight into Shiga toxin genes encoded by Escherichia coli O157 from whole genome sequencing. Peer J 3, e739

Assis FEA, Wolf S, Surek M, De Toni F, Souza EM, Pedrosa FO, Farah SMSS, Picheth G, Fadel-Picheth CMT (2014) Impact of Aeromonas and diarrheagenic Escherichia coli screening in patients with diarrhea in Paraná, southern Brazil. J Infect Dev Ctries 8:1609–1614

Barletta F, Ochoa TJ, Ecker L, Gil AI, Lanata CF, Cleary TG (2009) Validation of five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic Escherichia coli. J Clin Microbiol 47:1915–1917

Bertelli C, Greub G (2013) Rapid bacterial genome sequencing: methods and applications in clinical microbiology. Clin Microbiol Infect 19:803–813

Bläck E, Svennerholm AM, Holmgren J, Möllby R (1979) Evaluation of a ganglioside immunosorbent assay for detection of Escherichia coli heat-labile enterotoxin. J Clin Microbiol 10:791–795

Botkin DJ, Galli L, Sankarapani V, Soler M, Rivas M, Torres AG (2012) Development of a multiplex PCR assay for detection of Shiga toxin-producing Escherichia coli, enterohemorrhagic E. coli, and enteropathogenic E. coli strains. Front Cell Infect Microbiol 14:2–8

Brian MJ, Frosolono M, Murray BE, Miranda A, Lopez EL, Gomez HF, Cleary TG (1992) Polymerase chain reaction for diagnosis of enterohemorrhagic Escherichia coli infection and hemolytic-uremic syndrome. J Clin Microbiol 30:1801–1806

Buchan BW, Ledeboer NA (2014) Emerging technologies for the clinical microbiology laboratory. Clin Microbiol Rev 27:783–822

Buchan BW, Olson WJ, Pezewski M, Marcon MJ, Novicki T, Uphoff TS, Chandramohan L, Revell P, Ledeboer NA (2013) Clinical evaluation of a real-time PCR assay for identification of Salmonella, Shigella, Campylobacter (Campylobacter jejuni and C. coli), and Shiga toxin-producing Escherichia coli isolates in stool specimens. J Clin Microbiol 51:4001–4007

Bueris V, Palma Sircili M, Romano Taddei C, Fernandes dos Santos M, Franzolin MR, Baquerizo Martinez M, Ramos Ferrer S, Lima Barreto M, Trabulsi LR (2007) Detection of diarrheagenic Escherichia coli from children with and without diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 102:839–844

Caravelli A, Luz DE, Andrade FB, Moraes CT, Maranhão AQ, Piazza RM (2013) Sensitive and specific detection of enteropathogenic and enterohemorrhagic Escherichia coli using recombinant anti-intimin antibody by immunofluorescence assay. Diagn Microbiol Infect Dis 77:301–303

Centers for Disease Control and Prevention (CDC) (2009) Recommendations for diagnosis of Shiga toxin-producing Escherichia coli infections by clinical laboratories. MMWR Recomm Rep 58:1–12

Cerna JF, Nataro JP, Estrada-Garcia T (2003) Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative Escherichia coli strains. J Clin Microbiol 41:2138–2140

Chen Q, Shi X, Li Y, Jiang Y, Lin Y, Qiu Y, Li Q, Hu Q (2014) Rapid genetic typing of diarrheagenic Escherichia coli using a two-tube modified molecular beacon based multiplex real-time PCR assay and its clinical application. Ann Clin Microbiol Antimicrob 13:30

Chinen I, Rivas M, Caffer MI, Cinto RO, Binsztein N (1993) Diagnosis of Entero-Invasive Escherichia coli associated with diarrhea. Rev Argent Microbiol 25:27–35

Cravioto A, Tello A, Navarro A, Ruiz J, Villafán H, Uribe F, Eslava C (1991) Association of Escherichia coli HEp-2 adherence patterns with type and duration of diarrhea. Lancet 337:262–264

Dallman TJ, Byrne L, Ashton PM, Cowley LA, Perry NT, Adak G, Petrovska L, Ellis RJ, Elson R, Underwood A, Green J, Hanage WP, Jenkins C, Grant K, Wain J (2015) Whole-genome sequencing for national surveillance of Shiga toxin-producing Escherichia coli O157. Clin Infect Dis 61:305–312

De Carolis E, Vella A, Vaccaro L, Torelli R, Spanu T, Fiori B, Posteraro B, Sanguinetti M (2014) Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. J Infect Dev Ctries 8:1081–1088

Di Cristanziano V, Timmen-Wego M, Lübke N, Kaiser R, Pfister H, Di Cave D, Berrilli F, Kaboré Y, D'Alfonso R (2015) Application of luminex gastrointestinal pathogen panel to human stool samples from Côte d'Ivoire. J Infect Dev Ctries 9:884–889

Dudley EG, Thomson NR, Parkhill J, Morin NP, Nataro JP (2006) Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative Escherichia coli. Mol Microbiol 61:1267–1282

Ecker D, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, Hofstadler SA (2008) Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol 6:553–558

Escher M, Scavia G, Morabito S, Tozzoli R, Maugliani A, Cantoni S, Fracchia S, Bettati A, Casa R, Gesu GP, Torresani E, Caprioli A (2014) A severe foodborne outbreak of diarrhoea linked to a canteen in Italy caused by enteroinvasive Escherichia coli, an uncommon agent. Epidemiol Infect 142:2559–2566

European Centre for Disease Prevention and Control (2015) Expert opinion on the introduction of next-generation typing methods for food- and waterborne diseases in the EU and EEA. ECDC, Stockholm

Ewing WH (1986) The genus Escherichia. In: Edwards PR, Ewing WH (eds) Edwards and Ewing's identification of Enterobacteriaceae. Elsevier, New York, p 536

Ferdous M, Zhou K, de Boer RF, Friedrich AW, Kooinstra-Smid AM, Rossen JW (2015) Comprehensive characterization of Escherichia coli O104:H4 isolated from patients in the Netherlands. Front Microbiol 6:1348

Fialho OB, Souza EM, Dallagassa CB, Oliveira Pedrosa F, Klassen G, Irino K, Paludo KS, Assis FE, Surek M, Sousa Santos Farah SM, Fadel-Picheth CM (2013) Detection of diarrheagenic Escherichia coli using a two-system multiplex-PCR protocol. J Clin Lab Anal 27:155–161

Fleckenstein JM, Hardwidge PR, Munson GP, Rasko DA, Sommerfelt H, Steinsland H (2010) Molecular mechanisms of enterotoxigenic Escherichia coli. Microbes Infect 12:89–98

Foster MA, Iqbal J, Zhang C, McHenry R, Cleveland BE, Romero-Herazo Y, Fonnesbeck C, Payne DC, Chappell JD, Halasa N, Gómez-Duarte OG (2015) Enteropathogenic and enteroaggregative E. coli in stools of children with acute gastroenteritis in Davidson County, Tennessee. Diagn Microbiol Infect Dis 83:319–324

Franzolin MR, Alves RC, Keller R, Gomes TA, Beutin L, Barreto ML, Milroy C, Strina A, Ribeiro H, Trabulsi LR (2005) Prevalence of diarrheagenic Escherichia coli in children with diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 100:359–363

Freedman SB, Lee BE, Louie M, Pang XL, Ali S, Chuck A, Chui L, Currie GR, Dickinson J, Drews SJ, Eltorki M, Graham T, Jiang X, Johnson DW, Kellner J, Lavoie M, MacDonald J, MacDonald S, Svenson LW, Talbot J, Tarr P, Tellier R, Vanderkooi OG (2015) Alberta provincial pediatric EnTeric infection TEam (APPETITE): epidemiology, emerging organisms, and economics. BMC Pediatr 15:89

Gómez-Duarte OG, Bai J, Newell E (2009) Detection of Escherichia coli, Salmonella spp., Shigella spp., Yersinia enterocolitica, Vibrio cholerae and Campylobacter spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagn Microbiol Infect Dis 63:1–9

Gómez-Duarte OG, Arzuza O, Urbina D, Bai J, Guerra J, Montes O, Puello M, Mendoza K, Castro GY (2010) Detection of Escherichia coli enteropathogens by multiplex polymerase chain reaction from children's diarrheal stools in two Caribbean–Colombian cities. Foodborne Pathog Dis 7:199–206

Guth BEC, Picheth C, Gomes TAT (2010) Escherichia coli situation in Brazil. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 162–178

Hannaoui E, Villalobos L, Martínez R, Maldonado A, Hagel I, Bastardo J (2010) Diarrheagenic Escherichia coli associated with acute diarrhea in children of Cumaná, Venezuela. Invest Clin 51:489–500

Harrington SM, Buchan BW, Doern C, Fader R, Ferraro MJ, Pillai DR, Rychert J, Dayle L, Lainesse A, Karchmer T, Mortensen JE (2015) Multicenter evaluation of the BD Max enteric bacterial panel PCR assay for rapid detection of Salmonella spp., Shigella spp., Campylobacter spp. (C. jejuni and C. coli), and Shiga toxin 1 and 2 genes. J Clin Microbiol 53:1639–1647

Hayashimoto N, Morita H, Inoue T, Yasuda M, Yamamoto M, Itoh T (2015) Draft genome sequence of enteropathogenic Escherichia coli, isolated from the bloody stool sample of a common marmoset (Callithrix jacchus). Genome Announce 3:e01161-15

Hu Q, Lv D, Shi X, Jiang Y, Lin Y, Qiu Y, He L, Zhang R, Li Q (2014) Modified molecular beacons-based multiplex real-time PCR assay for simultaneous detection of eight foodborne pathogens in a single reaction and its application. Foodborne Pathog Dis 11:207–214

Iguchi A, Iyoda S, Seto K, Morita-Ishihara T, Scheutz F, Ohnishi M, Pathogenic E. coli Working Group in Japan (2015) Escherichia coli O-genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. J Clin Microbiol 53:2427–2432

Jenkins C, Chart H, Willshaw GA, Cheasty T, Smith HR (2006) Genotyping of enteroaggregative Escherichia coli and identification of target genes for the detection of both typical and atypical strains. Diagn Microbiol Infect Dis 55:13–19

Jenkins C, Dallman TJ, Launders N, Willis C, Byrne L, Jorgensen F, Eppinger M, Adak GK, Aird H, Elviss N, Grant KA, Morgan D, Mclauchlin J (2015) Public health investigation of two outbreaks of Shiga toxin-producing Escherichia coli O157 associated with consumption of watercress. Appl Environ Microbiol 81:3946–3952

Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM (2014) Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of Verotoxigenic Escherichia coli. J Clin Microbiol 52:1501–1510

Joensen KG, Tetzschner AMM, Iguchi A, Aarestrup FM, Scheutz F (2015) Rapid and easy in silico serotyping of Escherichia coli isolates by use of whole-genome sequencing data. J Clin Microbiol 53:2410–2426

Kagkli DM, Folloni S, Barbau-Piednoir E, Van den Eede G, Van den Bulcke M (2012) Towards a pathogenic Escherichia coli detection platform using multiplex SYBR green real-time PCR methods and high resolution melting analysis. PLoS One 7, e39287

Karmali MA, Steele BT, Petric M, Lim C (1983) Sporadic cases of hemolytic uremic syndrome associated with fecal cytotoxin and cytotoxin-producing Escherichia coli. Lancet 1(8325):619–620

Khare R, Espy MJ, Cebelinski E, Boxrud D, Sloan LM, Cunningham SA, Pritt BS, Patel R, Binnicker MJ (2014) Comparative evaluation of two commercial multiplex panels for detection of gastrointestinal pathogens by use of clinical stool specimens. J Clin Microbiol 52:3667–3673

Konowalchuk J, Speirs JI, Stavric S (1977) Vero response to a cytotoxin of Escherichia coli. Infect Immun 18:775–779

Kotloff KL, Blackwelder WC, Nasrin D, Nataro JP, Farag TH, van Eijk A, Adegbola RA, Alonso PL, Breiman RF, Faruque ASM, Saha D, Sow SO, Sur D, Zaidi AKM, Biswas K, Panchalingam S, Clemens JD, Cohen D, Glass RI, Mintz ED, Sommerfelt H, Levine MN (2012) The global enteric multicenter study (GEMS) of diarrheal disease in infants and young children in developing countries: epidemiologic and clinical methods of the case/control study. Clin Infect Dis 55:S232–S245

Lanata CF, Fischer-Walker CL, Olascoaga AC, Torres CX, Aryee MJ, Black RE (2013) Global causes of diarrheal disease mortality in children 5 years of age: a systematic review. PLoS One 8, e72788

Levine MM, Prado V, Robins-Browne R, Lior H, Kaper JB, Moseley SL, Gicquelais K, Nataro JP, Vial P, Tall B (1988) Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic Escherichia coli. J Infect Dis 158:224–228

Liew PS, Teh CSJ, Lau YL, Thong KL (2014) A real-time loop-mediated isothermal amplification assay for rapid detection of Shigella species. Trop Biomed 31:709–720

Lindsay B, Pop M, Antonio M, Walker AW, Mai V, Ahmed D, Oundo J, Tamboura B, Panchalingam S, Levine MM, Kotloff K, Li S, Magder LS, Paulson JN, Liu B, Ikumapayi U, Ebruke C, Dione M, Adeyemi M, Rance R, Stares MD, Ukhanova M, Barnes B, Lewis I, Ahmed F, Alam MT, Amin R, Siddiqui S, Ochieng JB, Ouma E, Juma J, Mailu E, Omore R, O'Reilly CE, Hannis J, Manalili S, Deleon J, Yasuda I, Blyn L, Ranken R, Li F, Housley R, Ecker DJ, Hossain MA, Breiman RF, Morris JG, McDaniel TK, Parkhill J, Sara D, Sampath R, Stine OC, Nataro JP (2013) Survey of culture, goldengate assay, universal biosensor assay, and 16S rRNA gene sequencing as alternative methods of bacterial pathogen detection. J Clin Microbiol 51:3263–3269

Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R, Haverstick DM, Houpt ER (2012) A laboratory-developed TaqMan array card for simultaneous detection of 19 enteropathogens. J Clin Microbiol 51:472–480

Lozer DM, Souza TB, Monfardini MV, Vicentini F, Kitagawa SS, Scaletsky ICA, Spano LC (2013) Genotypic and phenotypic analysis of diarrheagenic Escherichia coli strains isolated from Brazilian children living in low socioeconomic level communities. BMC Infect Dis 13:418

Melli LJ, Ciocchini AE, Caillava AJ, Vozza N, Chinen I, Rivas M, Feldman MF, Ugalde JE, Comerci DJ (2015) Serogroup-specific bacterial engineered glycoproteins as novel antigenic targets for diagnosis of Shiga toxin-producing- Escherichia coli-associated hemolytic-uremic syndrome. J Clin Microbiol 53:528–538

Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W, McLaughlin SF, Henkhaus JK, Leopold B, Bielaszewska M, Prager R, Brzoska PM, Moore RL, Guenther S, Rothberg JM, Karch H (2011) Prospective genomic

characterization of the German enterohemorrhagic Escherichia coli O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6, e22751
Moreno AC, Filho AF, Gomes TA, Ramos ST, Montemor LP, Tavares VC, Filho LS, Irino K, Martinez MB (2010) Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. Diagn Microbiol Infect Dis 66:50–57
Morin N, Santiago AE, Ernst RK, Guillot SJ, Nataro JP (2013) Characterization of the AggR regulon in enteroaggregative Escherichia coli. Infect Immun 81:122–132
Moyne A-L, Harris LJ, Marco ML (2013) Assessment of total and viable Escherichia coli O157:H7 on field and laboratory grown lettuce. PLoS One 8:e70643
Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, Edberg SC, Braden CR, Morris JG, Hirshon JM (2006) Diarrheagenic Escherichia coli infection in Baltimore, Maryland, and New Haven, Connecticut. Clin Infect Dis 43:402–407
Navarro A, Estrada-Garcia T (2010) Epidemiology of diarrheagenic Escherichia coli Pathotypes in Mexico, past and present. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 191–208
Nyholm O, Halkilahti J, Wiklund G, Okeke U, Paulin L, Auvinen P, Haukka K, Siitonen A (2015) Comparative genomics and characterization of hybrid shigatoxigenic and enterotoxigenic Escherichia coli (STEC/ETEC) strains. PLoS One 10:0135936
O'Ryan M, Prado V, Pickering LK (2005) A millennium update on pediatric diarrheal illness in the developing world. Semin Pediatr Infect Dis 16:125–136
Ochoa TJ, Barletta F, Contreras C, Mercado E (2008) New insights into the epidemiology of enteropathogenic Escherichia coli infection. Trans R Soc Trop Med Hyg 102:852–856
Ochoa TJ, Ecker L, Barletta F, Mispireta ML, Gil AL, Conteras C, Molina M, Amemiya I, Verastegui H, Hall ER, Cleary TG, Lanata CF (2009) Age-related susceptibility to infection with diarrheagenic Escherichia coli among infants from periurban areas in Lima, Peru. Clin Infect Dis 49:1694–1702
Ochoa TJ, Mercado EH, Durand D, Rivera FP, Mosquito S, Contreras C, Riveros M, Lluque A, Barletta F, Prada A, Ruiz J (2011) Frequency and pathotypes of diarrheagenic Escherichia coli in Peruvian children with and without diarrhea. Rev Peru Med Exp Salud Publica 28:13–20
Oh KH, Kim SB, Park MS, Cho SH (2014) Development of a One-step PCR assay with nine primer pairs for the detection of five diarrheagenic Escherichia coli types. J Microbiol Biotechnol 24:862–868
Ojima-Kato T, Yamamoto N, Suzuki M, Fukunaga T, Tamura H (2014) Discrimination of Escherichia coli O157, O26 and O111 from other serovars by MALDI-TOF MS based on the S10-GERMS method. PLoS One 9, e113458
Ørskov F, Ørskov I (1992) Escherichia coli serotyping and disease in man and animals. Can J Microbiol 38:699–704
Ørskov I, Wachsmuth IK, Taylor DN, Echeverria P, Rowe B, Sakazaki R, Orskov F (1991) Two new Escherichia coli O groups: O172 from "Shiga-like" toxin II-producing strains (EHEC) and O173 from enteroinvasive E. coli (EIEC). APMIS 99:30–32
Pál T, Pácsa AS, Emödy L, Vörös S, Sélley E (1985) Modified enzyme-linked immunosorbent assay for detecting enteroinvasive Escherichia coli and virulent Shigella strains. J Clin Microbiol 21:415–418
Panchalingam S, Antonio M, Hossain A, Mandomando I, Ochieng B, Oundo J, Ramamurthy T, Tamboura B, Zaidi AK, Petri W, Houpt E, Murray P, Prado V, Vidal R, Steele D, Strockbine N, Sansonetti P, Glass RI, Robins-Browne RM, Tauschek M, Svennerholm AM, Berkeley LY, Kotloff K, Levine MM, Nataro JP (2012) Diagnostic microbiologic methods in the GEMS-1 case/control study. Clin Infect Dis 55:S294–S302
Pattabiraman V, Bopp CA (2015) Draft whole-genome sequences of 10 enterotoxigenic Escherichia coli serogroup O6 strains. Genome Announce 3:e00564-15
Patzi-Vargas S, Zaidi MB, Perez-Martinez I, León-Cen M, Michel-Ayala A, Chaussabel D, Estrada-Garcia T (2015) Diarrheagenic Escherichia coli carrying supplementary virulence genes are an important cause of moderate to severe diarrhoeal disease in Mexico. PLoS Negl Trop Dis 9:e0003510

Pérez C, Gómez-Duarte OG, Arias ML (2010) Diarrheagenic Escherichia coli in children from Costa Rica. Am J Trop Med Hyg 83:292–297

Piazza RMF, Abe CM, Horton DSPQ, Miliwebsky E, Chinen I, Vaz TMI, Irino K (2010) Detection and subtyping methods of diarrheogenic Escherichia coli strains. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 95–115

Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR (1990) Rapid and specific detection of verotoxin genes in Escherichia coli by the polymerase chain reaction. J Clin Microbiol 28:540–545

Prasanna DK, Fisher MA (2013) Novel approach for differentiating Shigella species and Escherichia coli by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol 51:3711–3716

Qin X, Klein EJ, Galanakis E, Thomas AA, Stapp JR, Rich S, Buccat AM, Tarr PI (2015) Real-time PCR assay for detection and differentiation of Shiga toxin producing Escherichia coli from clinical samples. J Clin Microbiol 53:2148–2153

Ravan H, Amandadi M, Sanadgol N (2015) A highly specific and sensitive loop-mediated isothermal amplification method for the detection of Escherichia coli O157:H7. Microb Pathog 91:16–15

Regua-Mangia AH, Gomes TAT, Vieira MAM, Andrade JRC, Irino K, Teixeira LM (2004) Frequency and characteristics of diarrhoeagenic Escherichia coli strains isolated from children with and without diarrhoea in Rio de Janeiro, Brazil. J Infect 48:161–167

Reis MHL, Guth BEC, Gomes TAT, Murahovschi J, Trabulsi LR (1982) Frequency of Escherichia coli strains producing heat-labile toxin or heat-stable toxin or both in children with and without diarrhea in Sao Paulo. J Clin Microbiol 15:1062–1064

Rivas M, Moral LL, Binsztein N (1989) An evaluation of the pooling culture method for the detection of Escherichia coli enterotoxins. Rev Argent Microbiol 21:21–24

Rivas M, Padola NL, Lucchesi PMA, Masana M (2010) Diarrheogenic Escherichia coli in Argentina. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 142–161

Riveros M, Ochoa TJ (2015) Relevant public health enteropathogens. Rev Peru Med Exp Salud Publica 32(1):157–164

Rocha LB, Luz DE, Moraes CTP, Caravelli A, Fernandes I, Guth BEC, Horton DSPQ, Piazza RMF (2012) Interaction between Shiga toxin and monoclonal antibodies: binding characteristics and in vitro neutralizing abilities. Toxins 4:729–747

Rocha LB, Santos ARR, Munhoz DD, Cardoso LT, Luz DE, Andrade FB, Horton DS, Elias WP, Piazza RMF (2014) Development of a rapid agglutination latex test for diagnosis of enteropathogenic and enterohemorrhagic Escherichia coli infection in developing world: defining the biomarker, antibody and method. PLoS Negl Trop Dis 8, e3150

Rúgeles LC, Bai J, Martinez AJ, Vanegas MC, Gómez-Duarte OG (2010) Molecular characterization of diarrheagenic Escherichia coli strains from stools samples and food products in Colombia. Int J Food Microbiol 138:282–286

Rüttler ME, Yanzón CS, Cuitiño MJ, Renna NF, Pizarro MA, Ortiz AM (2006) Evaluation of a multiplex PCR method to detect enteroaggregative Escherichia coli. Biocell 30:301–308

Sánchez S, Llorente MT, Echeita MA, Herrera-León S (2015) Development of three multiplex PCR assays targeting the 21 most clinically relevant serogroups associated with Shiga toxin-producing E. coli infection in humans. PLoS One 10:e0117660

Scheutz F, Strockbine NA (2005) Escherichia. In: Garrity GM (ed) Bergey's manual of systematic bacteriology. Springer, New York, pp 607–624

Silva CJ, Erickson-Beltran ML, Skinner CB, Patfield SA, He X (2015) Mass spectrometry-based method of detecting and distinguishing type 1 and type 2 Shiga-like toxins. Toxins 7:5236–5253

Silveyra IM, Pereyra AM, Alvarez MG, Villagran MD, Baroni AB, Deza N, Carbonari CC, Miliwebsky E, Rivas M (2015) Isolation of enteropathogenic Escherichia coli O157:H16 identified in a diarrhea case in a child and his household contacts in La Pampa Province, Argentina. Rev Argent Microbiol 47:317–321

Tagg KA, Ginn AN, Partridge SR, Iredell JR (2015) MALDI-TOF mass spectrometry for multilocus sequence typing of Escherichia coli reveals diversity among isolates carrying blaCMY-2-like genes. PLoS One 10, e0143446

Toledo MRF, Trabulsi LR (1983) Correlation between biochemical and serological characteristics of Escherichia coli and results of the Sérény Test. J Clin Microbiol 17:419–421

Toledo MRF, Alvariza MCB, Murahovschi J, Ramos SRTS, Trabulsi LR (1983) Enteropathogenic Escherichia coli serotypes and endemic diarrhea in infants. Infect Immun 39:586–589

Torres ME, Pírez MC, Schelotto F, Varela G, Parodi V, Allende F, Falconi E, Dell'Acqua L, Gaione P, Méndez MV, Ferrari AM, Montano A, Zanetta E, Acuña AM, Chiparelli H, Ingold E (2001) Etiology of children's diarrhea in Montevideo, Uruguay: associated pathogens and unusual isolates. J Clin Microbiol 39:2134–2139

Varela G, Jazisnky C, Gadea P, Tanzi MN, Mota MI, Arenas C, Pardo L, González S, González G, Sirok A, Schelotto F (2007) Classic Enteropathogenic Escherichia coli (EPEC) associated with diarrhea in children users of the Hospital Pereira Rossell. Clinical aspects and characteristics of involved strains. Rev Med Urug 23:153–163

Varela G, Gómez-Garcia O, Ochoa T (2010) Diarrheogenic Escherichia coli in children from Uruguay, Colombia and Peru. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 209–222

Varela G, Batthyány L, Bianco MN, Pérez W, Pardo L, Algorta G, Robino L, Suarez R, Navarro A, Pírez MC, Schelotto F (2015) Enteropathogens associated with community-acquired acute diarrhea in children from households with high socio-economic level. Int J Microbiol 2015:592953

Vidal M, Kruger E, Duran C, Lagos R, Levine M, Prado V, Toro C, Vidal R (2005) Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic Escherichia coli associated with enteric infections. J Clin Microbiol 43:5362–5365

Vidal RM, Oñate A, Salazar JC, Prado V (2010) Shiga toxin producing Escherichia coli in Chile. In: Torres AG (ed) Pathogenic Escherichia coli in Latin America, vol 1. Bentham Science, Sharjah, pp 179–190

Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M (2002) N-linked glycosylation in Campylobacter jejuni and its functional transfer into E. coli. Science 298:1790–1793

World Health Organization (1987) Programme for control of diarrhoeal diseases (CDD/83.3 Rev 1). In: Manual for laboratory investigations of acute enteric infections. World Health Organization, Geneva, p 27

Yamasaki E, Watahiki M, Isobe J, Sata T, Nair GB, Kurazono H (2015) Quantitative detection of Shiga toxins directly from stool specimens of patients associated with an outbreak of enterohemorrhagic Escherichia coli in Japan—quantitative Shiga toxin detection from stool during EHEC outbreak. Toxins 7:4381–4389

Youmans BP, Ajami NJ, Jiang ZD, Petrosino JF, DuPont HL, Highlander SK (2014) Development and accuracy of quantitative real-time polymerase chain reaction assays for detection and quantification of enterotoxigenic Escherichia coli (ETEC) heat labile and heat stable toxin genes in travelers' diarrhea samples. Am J Trop Med Hyg 90:124–132

Index

A.G. Torres (ed.), *Escherichia coli in the Americas*,
DOI 10.1007/978-3-319-45092-6