Vaccines Against *Escherichia coli*

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Contents

Abstract *Escherichia coli* has a complex and versatile nature and continuously evolves from non-virulent isolates to highly pathogenic strains causing severe diseases and outbreaks. Broadly protective vaccines against pathogenic *E. coli* are not available and the rising in both, multi-drug resistant and hypervirulent isolates, raise concern for healthcare and require continuous efforts in epidemiologic surveillance and disease monitoring. The evolving knowledge on *E. coli* pathogenesis mechanisms and on the mediated immune response following infection or vaccination, together with advances in the "omics" technologies, is opening new perspectives toward the design and development of effective and innovative *E. coli* vaccines.

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1 Introduction

E. coli strains are classified into "pathotypes" (Kaper et al. 2004) and can be subtyped using a variety of criteria, including serotype, pulsotype, phage type, or biotype (Robins-Browne et al. 2016; Micenkova et al. 2016). *E. coli* can also be classified serologically on the basis of the O somatic antigen (Fratamico et al. 2016), K capsular polysaccharide surface antigen (Whitfield 2006; Kaczmarek et al. 2014), and H flagellar antigen (Geue et al. 2014; Chui et al. 2015). At the population level, *E. coli* can be phylogenetically assigned to five main groups: A, B1, B2, D, and E. Even if commensals mostly belong to the phylogroup A and B1, not all pathotypes group together, suggesting a disparate nature of pathogenic species (Leimbach et al. 2013).

The pathotypes of *E. coli* that are associated with intestinal disease are known collectively as intestinal pathogenic *E. coli* (IPEC) or diarrheagenic *E. coli* (DEC), while *E. coli* causing disease in tissues other than the intestinal tract are known collectively as extraintestinal pathogenic *E. coli* (ExPEC). ExPEC resides asymptomatically in the human intestinal tract of $\sim$20% of healthy individuals, sharing large genomic regions with nonpathogenic strains. In contrast to the facultative ExPEC pathogens which belong to the normal gut flora where they live as commensals, intestinal pathogenic *E. coli* (InPEC) are obligate pathogens epidemiologically and phylogenetically distinct from ExPEC and commensals.

Although all the classification schemes developed so far provide important information on the nature of the epidemiologically relevant strains, the whole genome analysis more accurately defines the differences in gene content and allelic variations among the different isolates allowing a more in-depth understanding of strain evolution and spreading.

The *E. coli* species undergo rapid genetic changes, referred to as microevolution, providing new traits, favoring the fitness and the adaptation to environmental changes (Brzuszkiewicz et al. 2009). Microevolutionary divergence is a common phenomenon in *E. coli*, as demonstrated by genomic studies on *E. coli* diversity (Moriel et al. 2012; Lo et al. 2015). Moreover, novel virulent isolates possessing hybrid features of different pathotypes are continuously causing emergent outbreaks worldwide. The 2011 outbreak in Germany was determined by an EAEC strain, which has acquired several mobile genetic elements including the phage-mediated Shiga Toxin Stx2a (Frank et al. 2011), opening new views on the designation of pathotypes (Brzuszkiewicz et al. 2011; Rasko et al. 2011). In addition, a growing number of studies are linking foodborne *E. coli* with uropathogenic strains. Thus, the term foodborne urinary tract infections (FUTIs) has been adopted to describe urinary tract infections (UTIs) with probable foodborne origins (Nordstrom et al. 2013).

E. coli is also rapidly evolving as multidrug-resistant bacterium, exacerbating the public health problems in the era of decline in antimicrobial drug discovery. The dangerousness of the prevalence of UPEC isolates resides in their resistance to the first-line oral antibiotic agents such as trimethoprim–sulfamethoxazole, ampicillin, and fluoroquinolones. In addition, the most common sources of infections consist of

fluoroquinolone-resistant strains colonizing the rectum and the urinary tract. In the recent years, the ST clonal group known as ST131, a virulent and epidemic antibiotic-resistant *E. coli*, caused severe hospital outbreaks with a strong potential for wide dissemination (Nicolas-Chanoine et al. 2014; Mathers et al. 2015). The increased extended-spectrum β-lactamase (ESBL)-producing *E. coli* by 300% is responsible for the growing burden and healthcare-related costs due to ExPEC and further highlights the urgent need for effective interventions (Blaak et al. 2014; Franz et al. 2015).

Today, the identification and tracking of multidrug-resistant microorganisms in hospitals and communities can be performed very rapidly by whole genome sequencing (Punina et al. 2015). The emerging and re-emerging infections and the spread of antibiotic resistance strains render the need for an effective vaccine able to prevent *E. coli* infection and disease a public health priority. Vaccination could represent a measure against antibiotic resistance spread by reducing the infection rate and as consequence, antimicrobial use (Lipsitch and Siber 2016).

1.1 ExPEC: Urinary Tract Infection, Neonatal Meningitis, and Sepsis

Extraintestinal pathogenic *E. coli* strains (ExPEC) include uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), septicemia-associated *E. coli* (SePEC), and avian pathogenic *E. coli* (APEC). UPEC infections account for >85% of cases of acute cystitis and pyelonephritis, >60% of recurrent cystitis. Infections that are not resolved with antibiotic prophylaxis could evolve in pyelonephritis, sepsis, or death. In the healthcare setting, catheter-associated UTIs (CAUTIs) represent the second most common cause of all nosocomial infections (1 million catheter-associated UTIs/year in the U.S.). UTIs are also a source of substantial morbidity in children under neonatal intensive care, where the risk of breakthrough UTIs (BUTIs) could occur during antibiotic treatments (Hidas et al. 2015; Lloyd et al. 2016).

E. coli is a leading cause of bacteremia worldwide (Laupland and Church 2014). The overall annual incidence of *E. coli* bacteremia in adults markedly increases with age, reaching 452 cases/100,000 person-years in individuals aged ≥ 85 years. Case fatality rates for bacteremia are between 13 and 19% but may be up to 60% in elderly persons with nosocomial infections (Roubaud Baudron et al. 2014) and neurological sequelae occur in 30–50% of cases (Logue et al. 2012). In addition, spread of *E. coli* bacteremia both in USA and in Europe is accompanied by a 30% annual increase in third-generation cephalosporin-resistant isolates (Carl et al. 2014; Basu 2015). Thus, *E. coli* bacteremia is a costly, potentially lethal, and increasingly frequent problem exacerbated by societal aging and increasing prevalence of antibiotic-resistant strains.

1.2 *Intestinal* E. coli *Infections*

The major intestinal pathotypes include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and adherent-invasive *E. coli* (AIEC).

ETEC produces both heat-labile (LT) and heat-stable (ST) enterotoxins and is the most important cause of watery diarrhea, with abdominal pains and vomiting, both in developing countries and in travelers endemic regions. ETEC is responsible for 280 million diarrheal episodes and more than 400 thousand death annually. In 2013, the World Health Organization (WHO) Child Health Epidemiology Reference Group estimated 42,000 (95% CI, 20,000–76,000) ETEC-associated deaths of children under five years of age (Lanata et al. 2013). Overall, ETEC causes approximately 10 million episodes of travelers' diarrhea each year, through Africa, Asia, and Latin America, including military personnel deployed to these areas. In addition, ETEC-associated travelers' diarrhea may go on to develop reactive arthritis, irritable bowel, and Guillain–Barré syndromes (Giddings et al. 2016).

Other intestinal *E. coli* pathotypes also contribute to diarrheal disease but can differ in terms of detection, diagnosis, epidemiology, public health, pathogenesis, and human disease. EPEC mainly affects small intestine of infant, causing diarrhea associated with fever, nausea, and vomiting, spreading an increased antibiotic-resistant strains in both developing and developed countries. EHEC affects large intestine causing severe abdominal pain, watery diarrhea followed by bloody diarrhea leading to hemolytic uremic syndrome. EIEC produces shigella-like diarrhea in large intestine and determines epithelial cells injury and tissue invasion. STEC is associated with a disease spectrum ranging from diarrhea and hemorrhagic colitis (HC) to the potentially fatal hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). EAEC, which affects small intestine, is responsible for endemic diarrhea of infants in both industrialized and developing countries.

2 Vaccines

Over the last two centuries, vaccination has been the most effective measure to save lives and improve public health. Conventional vaccinology, mainly based on the Pasteur's principles of inactivation of the disease agents and generation of killed or live attenuated vaccines, has experienced a deep renaissance, thanks to the understanding of virulence and immunity mechanisms and to the advent of new technologies of genetic engineering and of genomic sequencing and bioinformatics. *E. coli* vaccines proposed today are based on live attenuated strains rationally designed to be safe, with deletions in genes important for virulence and with improved immunogenicity, overexpressing selected antigens, or on whole inactivated strains and/or on new promising vaccine antigens discovered by proteomic and genomic approaches (Fig. 1).

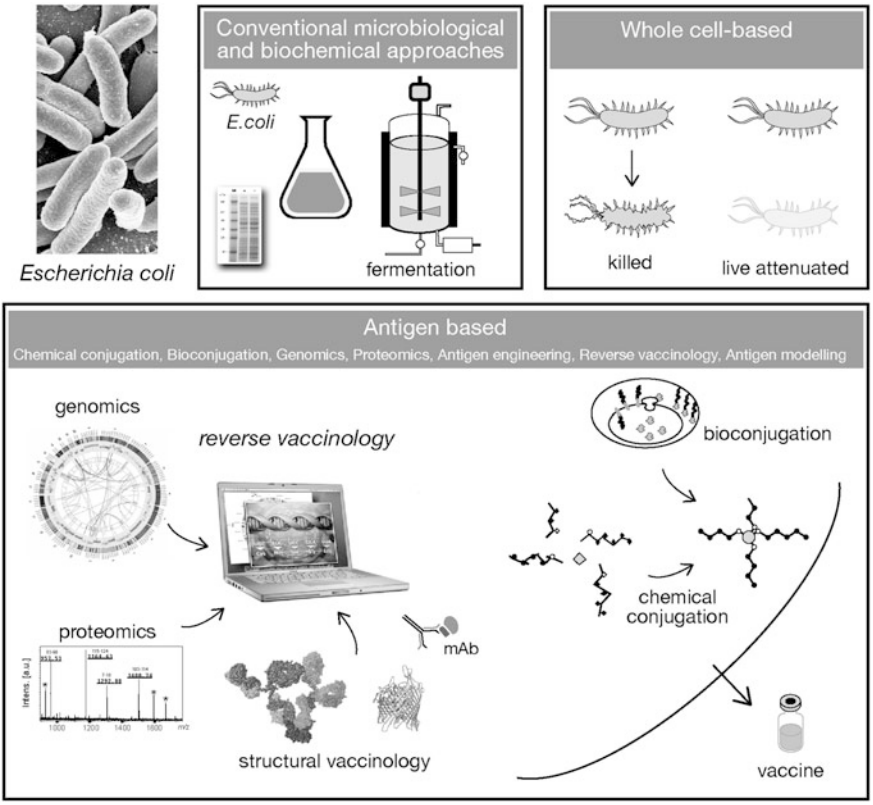

Fig. 1 Different strategies to vaccine discovery and development

2.1 Vaccines Against Extraintestinal Pathotypes

2.1.1 Conventional Vaccinology Against ExPEC

Conventional strategies applied to the development of an effective vaccine against ExPEC infections have been unsuccessful so far (Uehling and Wolf 1969; Kaijser et al. 1983a, b). In the 1990s, traditional vaccine strategies were based on single-purified virulence factors such as Hemolysin (O'Hanley et al. 1991) or on the O-specific polysaccharide (OPS) chain of the lipopolysaccharide (named O-antigen), conjugated to either *Pseudomonas aeruginosa* endotoxin A (TA) or cholera toxin (CT) as carrier proteins (Cryz et al. 1991; Cross 1994). Although the prevalence of capsular polysaccharide (K-antigen) and O-antigen is different among the different pathotypes, there is an association between K (K1, K5, 30 and 92) and O (O1, 2, 4, 6, 7, 8, 16, 16/72, 18, 25, 50 and 75) antigenic groups and uropathogenic strains (Brumbaugh and Mobley 2012). However, because of the high antigenic heterogeneity of the surface polysaccharides, the design of a

polysaccharide vaccine able to prevent ExPEC infections has been extremely challenging (Russo and Johnson 2006).

An O18-polysaccharide conjugated to either cholera toxin or to *P. aeruginosa* exoprotein A (EPA) was shown to be safe and able to induce antibodies with opsonophagocytic killing activity (OPK) in human volunteers. IgG purified from immunized individuals were protective in mice in an *E. coli* 018 challenge sepsis model (Cryz et al. 1991). When a 12-valent vaccine, based on O-antigen based on 12 serogroups of *E. coli* (O1, O2, O4, O6–O8, O12, O15, O16, O18, O25, O75) conjugated to EPA, was tested in a clinical trial, the functional immunoresponse induced by each O-antigen was different, underlying the difficulties of development of a cross-protective vaccine (Cross et al. 1994).

Vaccines based on whole or lysed fractions of inactivated *E. coli* have been evaluated in human clinical trials (Fig. 2) and have been so far the most effective in inducing some degree of protection in subjects undergoing recurrent urinary tract infections. The sublingual vaccine Uromune, an inactivated whole preparation of *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Enterococcus faecalis*, evaluated as prophylactic treatment in a multicenter retrospective observational study, demonstrated a certain degree of clinical benefit in terms of reduced recurrence rate in women with a history of recurrent UTI (Lorenzo-Gomez et al. 2013). The Solco Urovac vaccine, a vaginal suppository polymicrobial vaccine consisting of 10 inactivated uropathogenic bacteria, including 6 *E. coli* serotypes, *Proteus mirabilis*, *Morganella morganii*, *K. pneumoniae,* and *E. faecalis* strains, showed a minimal efficacy in Phase 1 and two Phase 2 trials in women suffering of recurrent UTIs (Uehling et al. 2001, 2003; Bauer et al. 2005). However, in two additional clinical studies, the vaginal mucosal vaccine given for a 14-week period increased the time to re-infection in UTI susceptible women, representing a valuable alternative to the antibiotic-based prophylactic regimens (Uehling et al. 2003; Hopkins et al. 2007). A post-marketing assessment further demonstrated the significant reduction of Solco-Urovac on recurrent UTIs when administered as standalone or in conjunction with standard antibacterial medications (Kochiashvili et al. 2014).

Human trials with a vaccine based on *E. coli* Extract (ECE) started in the 1980s (Frey et al. 1986; Tammen 1990; Magasi et al. 1994), and efficacy and safety of *E. coli* extract (ECE; Uro-Vaxom) were assessed in larger clinical trials a few years later (Bauer et al. 2005; Kim et al. 2010) leading to the recommendation of Uro-Vaxom for prophylactic treatment of patients with recurrent urinary tract infections. Additional studies, based on oral tablet, OM-89/Uro-Vaxom vaccine, demonstrated modest protection in women (Bauer et al. 2002). However, in a more recent trial on 451 female subjects, the lyophilized lysate of 18 *E. coli* strains, OM-89/Uro-Vaxom, manufactured using a modified lytic process, based on alkaline chemical lysis and autolysis, failed to show a preventive effect on recurrent uncomplicated UTIs (Wagenlehner et al. 2015).

Among the variety of strategies to develop a vaccine targeting ExPEC, those based on wild-type or genetically engineered inactivated uropathogens combination also resulted in weak success (Schmidhammer et al. 2002). Unfortunately, although

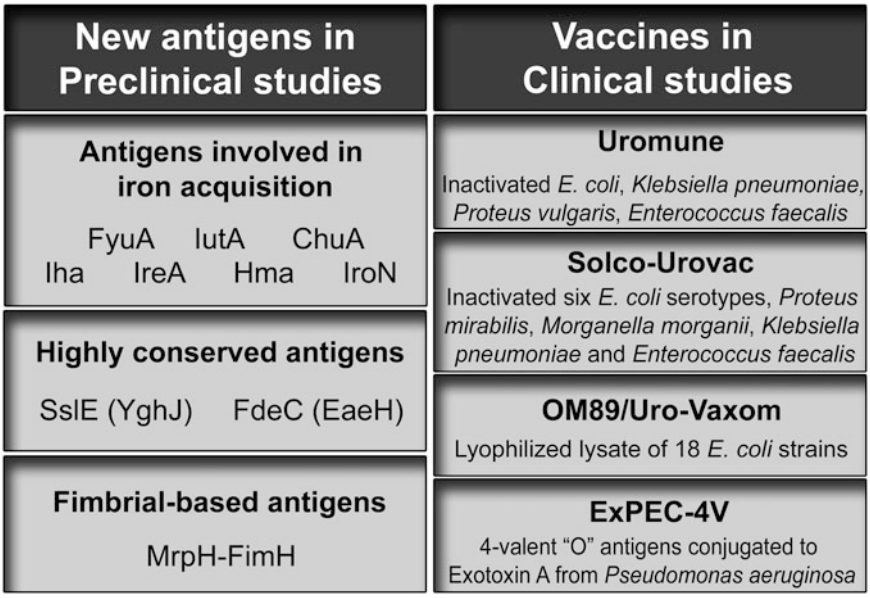

Fig. 2 Vaccines against Extraintestinal *E.coli* in clinical and preclinical studies

promising in preclinical studies, most of the vaccines based on these approaches failed to provide protection in clinical trials.

Regarding the use of single antigens, the most relevant that has been explored for its vaccine potential over the past 17 years is the Type 1 fimbrial adhesin FimH, which mediates UPEC adherence to bladder epithelial cells. The parenteral FimCH vaccine, composed of the FimH adhesin, the minor component of the Type 1 pili, in complex with its chaperone FimC, reduced bladder colonization in a mouse model, and induced protection from bladder infection and from inflammatory response in a monkey's model when used in combination with MF59 as systemic adjuvant (Langermann et al. 2000; Langermann and Ballou 2001). The data from the FimCH Phase II clinical trials have not been published yet and the level of efficacy of the FimCH vaccine is still unknown.

2.1.2 Emerging Approaches for Vaccines Against ExPEC

Chemically conjugated *E. coli* O-antigen vaccines are safe and immunogenic in humans; however, as discussed previously, production of multiple O-conjugates has been technically difficult. Very recently, an innovative technology, based on bio-conjugation, a process of in vivo synthesis, and conjugation of polysaccharide structures to carrier proteins in engineered bacterial cells, has been developed and shown to be a valuable approach for the production of multivalent conjugated vaccines. In the case of the ExPEC vaccine, the protein glycosylation machinery of

E. coli has been used to produce a conjugate vaccine based on a genetically detoxified form of *P. aeruginosa* exotoxin A (EPA) linked to the O1, O2, O6, and O25 *E. coli* serotype surface polysaccharide antigens. The immunogenicity and safety of the 4-valent "O" antigen bioconjugate *E. coli* vaccine (ExPEC-4V) have been evaluated in a phase 1b trial on healthy adult women with a history of recurrent UTIs. The vaccine was well tolerated and able to elicit functional antibody responses against all vaccine serotypes (Huttner et al. 2017). Phase II clinical trials are ongoing.

With the advent of the "omics" era, including genomic, proteomic, and transcriptomic, many new potential antigens have been identified and their protective properties tested in a variety of animal models (Fig. 2). UPEC strains survive in iron-limited conditions by upregulating the expression of iron acquisition systems. In 2010, putative UPEC-specific vaccine targets antigens involved in iron acquisition were identified: FyuA, IutA, ChuA, Hma, IhaA, IreA, and IroN (Durant et al. 2007; Alteri et al. 2009; Wieser et al. 2010; He et al. 2010). The newly identified antigens elicited a protective systemic and mucosal immune response in mice immunized intranasally being able to significantly reduce bladder and/or kidneys colonization (Alteri et al. 2009). These antigens have an important role in pathogenesis acting as siderophore receptors and meet all criteria for an antigen to be a potential vaccine candidate, including surface accessibility, recognition by the host immune system, in vitro expression in bacteria grown in human urine or in experimental conditions mimicking the urinary tract or the bladder environment, and in vivo expression in challenged mice and in women with UTI infections (Sivick and Mobley 2009; Vigil et al. 2011). Vaxign, a web-based vaccine design program, which contains prediction of vaccine targets for >70 genomes, has been used to predict new UPEC vaccine candidates based on the reverse vaccinology approach, successfully applied to the discovery of a new MenB vaccine (Pizza et al. 2000; Rappuoli 2001a, b). Vaxign predicts antigens on the basis of their subcellular localization, the presence of transmembrane helices, adhesin probability, low conservation to human and/or mouse proteins, the absence in genome(s) of non-pathogenic strain(s), and epitope binding to MHC class I and class II molecules (He et al. 2010). The selection criteria applied by H. Mobley's group in identifying the most promising candidates have been pivotal to reduce the number of potential vaccine antigens to be tested and allowed the selection of only six vaccine candidates for a single uropathogenic strain (Mobley and Alteri 2015). This approach highlights the importance of the basic knowledge in the virulence mechanisms as antibodies raised by the selected antigens are expected to interfere with the most critical steps of *E. coli* virulence and pathogenesis.

In 2010, a number of potential vaccine candidates against ExPEC were identified using the so-called "subtractive reverse vaccinology" approach, based on the genome comparison of three ExPEC strains (CFT073, 536, and IHE3034 to MG1655, DH10B, and W3110 nonpathogenic *E. coli* strains). By this approach, 230 potential antigens were identified and tested in a mouse model of sepsis, and nine of them were found to be protective (Moriel et al. 2010). Two of the newly identified antigens, ECOK1_0290 (FdeC, the Factor Adherence *E. coli*) and

ECOK1_3385 (SslE, Secreted and surface-associated lipoprotein from *E. coli*), were further analyzed for their protective ability in different animal models, functional and structural properties, in vivo expression during infection, and molecular epidemiological features. Mucosal immunization with the recombinant FdeC, deriving from an NMEC strain, using the cholera toxin (CT) as adjuvant, provided considerable protection in the ascending UTIs mouse model by challenge with two different UPEC disease isolates, supporting also its cross-protective ability. Of interest, FdeC conferred site-specific protection, as immunized mice were significantly protected from uropathogenic strains ascending toward the kidney, with a 1.5–2.5 log in median CFU/g range of reduction in kidney colonization. The high conservation of FdeC among strains belonging to different *E. coli* pathotypes, consisting of 99% gene presence in extraintestinal and 93–100% in intestinal pathotypes, and amino acid sequence identity >91% among all pathotypes, highlights the potential use of FdeC as a component of a broadly protective vaccine against extraintestinal and intestinal *E. coli* infections (Nesta et al. 2012).

Secondary structure prediction on FdeC, confirmed by the X-ray structure of the central domain, revealed an interesting structural similarity with the *Yersinia pseudotuberculosis* invasin and EPEC intimin. In agreement with the prediction, the recombinant FdeC protein demonstrated a strong affinity in binding to several epithelial cell lines in vitro and to specifically target different collagen types, including type V and VI, both widely expressed in the interstitial space of kidney and bladder. However, an intriguing FdeC peculiarity is that its expression on the bacterial surface is triggered upon interaction of an NMEC K1 strain with host cells in vitro. Interestingly, FdeC expression was detected in vivo, on UPEC strains closely associated with bladder tissues of mice following intraurethral challenge. In vivo competition experiments between UPEC wild-type and its derivative *fdeC* mutant revealed that the loss of FdeC caused a significant reduction in bacterial fitness (Nesta et al. 2012). In agreement, the ETEC *eaeH* gene, the homolog of ExPEC *fdeC*, was found significantly upregulated upon host cell contact (Kansal et al. 2013) and the expression of EaeH in ETEC was also demonstrated during pathogen–epithelial cell interaction in vitro (Sheikh et al. 2014). These findings are consistent with the hypothesis that this protein is activated by and participates in intimate interactions of both ETEC and ExPEC with the target epithelium. The indications on the structural and functional role of FdeC in bacterial pathogenesis and tissue adhesion may suggest that antibodies against FdeC could reduce colonization.

Ability of SslE to act as protective antigen against ExPEC infections has been confirmed in different animal models, using different clinical isolates as challenge strains (Moriel et al. 2010). In the UTI mouse model, intranasal immunization with SslE, using cholera toxin as mucosal adjuvant, led to a significant reduction of bacterial load in the kidneys and a more pronounced in the spleen with a 2.0 log reduction in median CFU/g following intraurethral challenge with the UPEC strain. In the sepsis model, SslE determined a significant protection from mortality (60% survival, $P < 0.0001$) against a SEPEC challenge strain, expressing a distant SslE variant. In addition, SslE was able to induce protection in terms of 1 log reduction

in bacterial load in the intestine of mice challenged orally with an ETEC strain, reinforcing the potential of this antigen as universal *E. coli* vaccine candidate (Moriel et al. 2010; Nesta et al. 2014).

By the functional point of view, SslE is the substrate of a T2SS (Type 2 Secretion System) and an outer membrane lipoprotein also known as YghJ in the case of ETEC (Yang et al. 2007; Iguchi et al. 2009). It has been associated with the M60-like extracellular zinc-metalloprotease subfamily, implicated in glycan recognition and processing. Functional activity of SslE has been controversial since it was originally shown to be involved in biofilm formation of an EPEC strain (Baldi et al. 2012), but this function was not confirmed in subsequent studies (Hernandes et al. 2013). More recently, the functional activity of SslE as a mucinase enzyme has been elucidated using a variety of in vitro methods (Nesta et al. 2014; Valeri et al. 2015). Of interest, an in vitro assay specifically set up to quantify the bacterial mucinase activity by counting the number of bacteria able to traverse an agar-based mucin matrix was used both to demonstrate SslE activity and, most importantly, to evaluate the ability of antibodies raised by immunization with SslE to inhibit mucinase activity. Interestingly, antibodies raised against an ExPEC SslE variant were able to specifically inhibit the mucinase activity of different *E. coli* pathotypes expressing distant SslE variants, including EPEC, SEPEC, ETEC, and the EAHEC strain responsible for the 2011 German outbreak, highlighting the potential role of this antigen as cross-protective against different pathotypes. On the basis of the functional role, it can be hypothesized that SslE may facilitate bacterial penetration of the mucosal surface and of the inner mucus layer, to allow *E. coli* to reach the underlying host epithelium.

Additional antigens have recently been shown to elicit protection in mouse model. Among them, the common pilus antigens EcpA and EcpD (*E. coli* common pilus, ECP) and iron uptake proteins IutA and IroN have been described as able to induce high levels of total IgG antibody of IgG1/IgG2a isotypes and to be protective in active and passive immunizations in a mouse model of sepsis (Mellata et al. 2016). Moreover, antibodies raised against a synthetic form of a conserved surface polysaccharide, β-(1-6)-linked poly-N-acetylglucosamine (dPNAG) containing nine monomers of (non-acetylated) glucosamine (9GlcNH2) conjugated to tetanus toxoid TT (9GlcNH2-TT) were shown to increase the efficacy of the passive immunization. These promising data represents an additional step toward the development of a broadly protective intervention against sepsis caused by *E. coli* (Mellata et al. 2016).

A recently proposed vaccine against UTIs is based on the immunogenic and protective MrpH-FimH fusion protein, made by MrpH from *P. mirabilis* and type 1 fimbrial FimH adhesin from a uropathogenic *E. coli* strain. Transurethral immunization of mice with the MrpH-FimH fusion induced a significant decrease in the number of bacteria recovered from bladder and kidney following challenge with UPEC or *P. mirabilis* strains, demonstrating the potential of MrpH-FimH as a promising vaccine candidate against UTIs caused by both UPEC and *P. mirabilis* (Habibi et al. 2016).

2.2 Vaccines Against Intestinal Pathotypes

2.2.1 Vaccines Against ETEC

Currently, there are no licensed vaccines against ETEC. Human challenge studies indicate that protective immunity against ETEC is induced after natural or experimental infection, suggesting that the development of an effective vaccine is feasible. Main efforts in developing vaccines against ETEC have been based on the induction of antitoxin and/or anti-colonization immunity (Fig. 3). Inhibition of ETEC adhesion to intestinal epithelial cells and neutralization of the toxic activity of the toxins should allow prevention of infection and disease. The only ETEC vaccine shown to provide some protection against diarrhea is the whole-cell vaccine containing the protective B subunit of the cholera toxin (CT-B), antigenically similar to the ETEC heat labile toxin (LT). The Dukoral vaccine, designed and licensed to prevent cholera, is in fact recommended to people visiting endemic regions, to prevent travelers' diarrhea.

ETEC bacteria use plasmid-encoded fimbrial colonization factors (CFs) or *E. coli* surface antigens (CS) to bind to enterocytes in the upper small intestine. Following preliminary colonization, the bacteria produce heat-stable (ST) and/or heat-labile (LT) enterotoxins that stimulate the release of fluid and electrolytes from the intestinal epithelium, resulting in the watery diarrheal illness. These plasmid-encoded antigens are known to be key virulence factors and have been proposed as vaccine components over the last three decades.

The LT enterotoxin is an ADP-ribosylating toxin, consisting of an enzymatically active A subunit non-covalently linked to a pentameric B subunit mediating the binding to host receptors, with strong immunogenic and adjuvant properties. Genetically detoxified derivatives of LT, devoid of toxicity but retaining the immunologic and adjuvant properties of the wild-type toxin, have been generated and extensively characterized in many animal models (Giuliani et al. 1998; Pizza et al. 2001; Norton et al. 2011). The majority of ETEC vaccine studies conducted so far only include LT-B as immunogen, but in more recent studies the A subunit is also included, based on the important contribution of LTA on the quality of the immune response in terms of IgG1/IgG2 balance and mucosal IgA and IL-17 secretion (Norton et al. 2012; Norton et al. 2015) and of the full toxin in inducing protective immunity (Giuliani et al. 1998). Genetically detoxified LT mutants are included in the newly proposed ETEC vaccine formulations (Zhang and Sack 2015).

Efficacy and safety of a skin patch vaccine containing the heat-labile toxin (LT) in travelers to Mexico and Guatemala have been assessed in phase 3 clinical trial. The transcutaneous LT-based ETEC vaccine failed in inducing protection against diarrhea in travelers, although the LT antigen was delivered effectively by skin immunization (Behrens et al. 2014). Because of these data, the use of the LT patches has no longer been considered a suitable approach for vaccination against ETEC (Riddle and Savarino 2014).

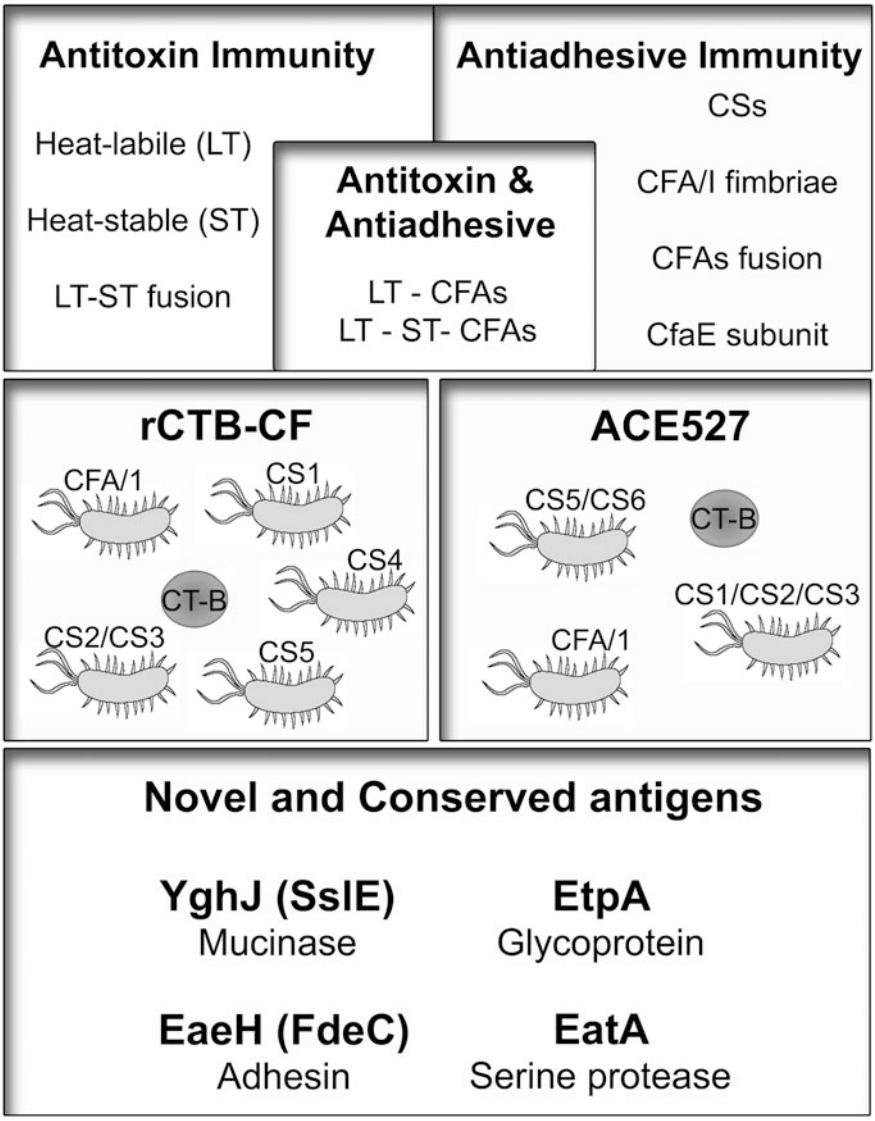

Fig. 3 New approaches to antigen-based vaccines against Enterotoxigenic *E.coli*

In contrast to LT, the reduced immunogenicity and the potent toxicity of STa has been an obstacle for many years to the development of toxoid-based vaccines against ETEC. ST consists of 18 (STp) or 19 (STh) amino acids and includes six cysteines that form three intramolecular disulfide linkages. Due to its small size, ST is nonimmunogenic in its natural form but becomes immunogenic when coupled to large-molecular-weight carrier, either by chemical conjugation or recombinant

fusion. Many genetic approaches have been explored to attenuate ST toxicity while enhancing its immunogenicity, considering that an ST toxoid-containing vaccine may cover potentially a broad range of ETEC infections (Taxt et al. 2010; Ruan et al. 2014).

ETEC colonization factors (CFs), categorized as colonization factor antigens (CFA) or *E. coli* surface antigens (CS), have been proposed as vaccine components, even if their distribution among ETEC is variable. CFA/I, CS3, CS5, and CS6 account for 50–80% of all CF-positive clinical ETEC isolates and some CF/CS antigens are immunologically related to the more prevalent CFs (i.e., CFA/I and CS14). In order to specifically abrogate the initial step of ETEC colonization, alternative approaches that target the CFA/I fimbriae or its CfaE tip-localized adhesin have been evaluated in preclinical animal models (Luiz et al. 2015). In addition, the ETEC fimbrial adhesin-based vaccine approach has been supported by further studies conducted both in mice and in nonhuman primates (Sincock et al. 2016). A novel multiepitope fusion antigen (MEFA) strategy has been recently used to construct ETEC fusion antigens starting from CFA adhesins, combined with or fused to an LT-STa toxoid fusion (Ruan et al. 2015).

In ETEC, there are more than 20 CFAs expressed in different combinations and in different geographic regions. However, a candidate vaccine formulated to cover CFA/I, CS3, and CS6 would only provide coverage for approximately 50–60% of the ETEC strains. To this end, inclusion of an LT toxoid in a CF-based vaccine may help to provide the potential vaccine strain coverage for LT-only strains that lack CF/CS selected antigens. Overall, optimal combination of toxin and CFAs for a specific target population may not always be easy. Consequently, additional antigens would need to be included to meet optimal vaccine coverage thresholds.

The rCTB-CF ETEC vaccine, composed of five formalin-killed *E. coli* strains expressing CFA/1, CS1, CS2/CS3, CS4, and CS5 adhesins, together with the recombinant B subunit of the cholera toxin, was tested in safety and immunogenicity in different trials, including adult volunteers from endemic areas such as Egypt and Israeli (Cohen et al. 2000), but did not reduce the overall rates of travelers' diarrhea (Sack et al. 2007). A phase 2b study of an oral, live attenuated, three-strain recombinant vaccine, ACE527, which expresses the colonization factors (CFs) CFA/I, CSI, CS2, CS3, CS5, and CS6 and heat-labile toxin B subunit (LT-B) induced clinically significant attenuation of diarrheal illness and reduced ETEC intestinal colonization in a stringent ETEC H10407 human challenge model (Darsley et al. 2012). A vaccine designed to specifically target ETEC, consisting of both killed whole cells and the recombinant CT-B, did not demonstrate clinically important benefits in two trials of 799 people traveling from the USA to Mexico or Guatemala, and from Austria to Latin America, Africa, or Asia, but was associated with increased vomiting (Ahmed et al. 2013c). A novel multicomponent oral inactivated whole-cell ETVAX vaccine, adjuvanted with an attenuated double-mutant form of LT (dmLT), was developed for both pediatric and traveler's indication. A phase I/II trial indicated that the addition of dmLT further enhanced mucosal immune responses to CF antigens present in low amounts in the vaccine as well as toxin-neutralizing antibody response to LT toxin (Lundgren et al. 2014).

In a recent study in which human volunteers were challenged or re-challenged with virulent ETEC strain H10407 serotype O78:H11, novel immunological benchmarks for the evaluation of ETEC vaccines were established as IgA responses to lipopolysaccharide (LPS), heat-labile toxin B subunit (LTB), and colonization factor antigen I (CFA/I) in lymphocyte supernatant (ALS), feces, lavage fluid, and saliva samples (Chakraborty et al. 2015). Overall, a limited number of ETEC vaccine trials conducted among younger age groups in endemic areas indicated that many questions still remain to be addressed to determine the vaccines impact against more severe or life-threatening ETEC disease (Das et al. 2013). Despite the significant effort in ETEC vaccine trials, these formulations have not been particularly effective in mediating cross-protective immunity.

Together with traditional approaches based on CFA and toxins, putative conserved pan-ETEC antigens have been also considered as promising vaccine candidates (Fig. 3).

Functional studies on the two-partner secretion system demonstrated that the secreted glycoprotein EtpA acted as a molecular bridge between ETEC flagellin and host cell receptors (Roy et al. 2009). Antibodies directed against either EtpA or the conserved regions of flagellin inhibited toxin delivery in vitro and prevented ETEC intestinal colonization in preclinical experiments. Of interest, mice intestinal colonization was significantly impaired using LT together with EtpA, suggesting the potential of EtpA as vaccine component (Roy et al. 2012).

The EaeH adhesin, the product of the *eaeH* gene first identified by subtractive hybridization of ETEC (Chen et al. 2006), is a conserved outer membrane protein that promotes bacterial engagement with host epithelial cell surfaces and ETEC colonization of the host's small intestine (Sheikh et al. 2014). The data on the EaeH adhesin, also known as FdeC in ExPEC, further support previous evidences on the crucial role of this antigen during bacterial colonization and highlight its potential as a component of a broadly protective vaccine against pathogenic *E. coli* (Nesta et al. 2012).

A chimeric vaccine containing the B subunit of heat labile toxin (LT-B) and the major subunit of CS3 was able to elicit high antibody titers in mice and to reduce ETEC adhesion to intestinal cells in vitro (Alerasol et al. 2014).

Proteins involved in mucin degradation were also proven as vaccine against ETEC in preclinical studies. EatA is a member of the serine protease autotransporter family of virulence proteins degrading MUC2, a major component of intestinal mucin. Of interest, antibodies against a secreted passenger domain of EatA were shown to impair the ETEC colonization of small intestine in mice (Luo et al. 2014). In agreement with evidences on SslE mucinase activity (Nesta et al. 2014), functional studies demonstrated that ETEC YghJ was specifically involved in degradation of mucin substrates, including Muc2 and Muc3 and required for efficient delivery of heat-labile toxin (Luo et al. 2014). In addition, SslE is present and conserved also among intestinal pathotypes, with an overall amino acid sequence identity ranged from 86 to 100%. The SslE heterologous and intrinsic protection against ETEC was assessed in a mouse model of intestinal colonization, resulting in a statistically significant 2.5 log reduction in the mean value of bacterial counts in

the caecum of immunized mice. In addition, protected mice developed anti-SslE antibodies belonging to both IgG and IgA isotypes, supporting the mucosal immunization as efficacious delivery, and reinforcing the potential of this antigen to broadly target pathogenic *E. coli* (Nesta et al. 2014).

Recently, antisera against a number of ETEC proteins that differed in their abundance in membrane protein preparations from wild-type versus a type II secretion mutant of ETEC, were tested in the ability to prevent ETEC adherence to cultured intestinal epithelial cells. Three of these antigens, ETEC_2479, Skp and MipA, were also able to provide a protective immunity in an intranasal mouse challenge model (Kumar et al. 2015).

2.2.2 Multivalent ETEC Vaccines

In the future, research will also be directed toward combining monovalent vaccines in a single complex vaccine to offer broad-spectrum coverage against different pathogens for the same target populations. The Global Enteric Multicenter Study (GEMS) revealed that *Shigella* and ETEC are among the top five major causes of moderate to severe diarrhea in children under 5 years of age in Africa and Asia (Kotloff et al. 2013). Among the many causes of diarrheal disease among travelers, military personnel visiting endemic areas, infants in developing countries, ETEC and *Shigella* are the two most important bacterial pathogens for which there are no currently licensed vaccines. Then, many attempts have been dedicated to achieve the goal of an immunogenic bivalent ETEC/*Shigella* vaccine. A potential attractive strategy is based on the use of attenuated strains of *Shigella* as live vectors for the expression of ETEC antigens, including CFs and mutant forms of LT (Ranallo et al. 2005; Barry et al. 2006). Even if an ETEC/*Shigella* is an evident option, other combinations may be considered. Since rotavirus and ETEC are of greatest threat to younger children, a combination vaccine against these may be an attractive approach. On the other hand, *Vibrio cholerae* and ETEC remain a massive burden in developing countries with increasing morbidity and mortality rates. Approaches aimed to target these two diarrhea-causing agents have been analyzed in preclinical studies. Immunization with a mixture of detoxified and enterotoxin-negative outer membrane vesicles (OMVs) derived from *V. cholerae* and ETEC induced a protective immune response against both pathogens (Leitner et al. 2015).

2.2.3 Vaccines Against Shiga Toxin-Producing *E. coli*

Shigatoxigenic *E. coli* (STEC), also referred to as verocytotoxin-producing *E. coli* (VTEC), are strains which produce Stx1 and Stx2 Shiga toxins, also known as verotoxins. Specific to their toxin-producing capabilities, VTEC and STEC *E. coli* nomenclature commonly refers to strains within the enterohaemorrhagic (EHEC) pathotype. The *E. coli* carrying both the Shiga toxin and intimin, the adhesive protein encoded by the *eae* gene and responsible for bacterial attaching to the

intestinal wall, commonly known as EHEC, elaborate potent Shiga toxins (Stx1 and/or Stx2) and are implicated in the development of hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS).

A fusion protein composed of the B subunits of the two types of Stx (named 2S protein) generated antibodies able to neutralize the cytotoxic activity of both Shiga toxins in vitro and to increase the survival of mice challenged with a lysate of *E. coli* O157:H7 (Gao et al. 2009). *Salmonella enterica* strains expressing the Stx2ΔAB toxoid colonized the mice gut and induced anti-Stx2B IgG. The anti-toxoid antibodies neutralized Stx2 toxic activity in vitro, but conferred only limited protection against the Stx2 challenge in vivo (Rojas et al. 2010). An S2 derivative fusion containing an enzymatically inactive Stx2A subunit instead of the Stx2B (Stx2Am-Stx1B) displayed enhanced immunogenicity compared to the S2 fusion. Stx2Am-Stx1B generated higher levels of Stx2 neutralizing antibodies and significantly higher level of protection against a lethal dose of an O157:H7 lysate (Cai et al. 2011). A vaccine containing a synthetic monomer of PNAG (9GlcNH2) conjugated to Shiga toxin 1b subunit was recently proposed to prevent intestinal infections caused by Shiga toxin (Stx)-producing *E. coli* (STEC) (Gould et al. 2013).

A number of EHEC vaccine approaches have been employed with different outcomes in animal models, including the use of recombinant proteins and virulence factors such as Stx1/2, intimin, EspA, fusion proteins of A and B Stx subunits, avirulent ghost cells of EHEC O157:H7, live attenuated bacteria expressing recombinant proteins, and recombinant fimbrial proteins (Rabinovitz et al. 2012; McNeilly et al. 2015). In order to prevent EHEC, vaccination of infected mice with a Stx2 toxoid resulted in decreased CFU detected in their feces, suggesting that active immunization leads to the generation of Stx2-neutralizing antibodies in the intestine (Mohawk et al. 2010). An EHEC multi-antigen vaccine consisted of the multivalent Stx2B-Tir-Stx1B-Zot protein, where Zot is used as an antigen delivery tool that binds a receptor in the intestinal epithelium affecting mucosal permeability. Mice immunized intranasally with this multivalent protein had reduced colonization and reduced amounts of EHEC detected in the stool (Zhang et al. 2011).

It is known that naturally occurring human EHEC O157:H7 infections induce antibodies against T3SS-related proteins, such as Tir, intimin, EspB, NleA. and EspA (Asper et al. 2011). This has led to the proposal of bacterial T3SS proteins (TTSPs) as vaccine candidates. Intestinal mucosa immune responses have also been targeted by intimin-, EspB-, and EspA-derived vaccines using different carrier strains. *Lactobacillus lactis* expressing EspA or EspB induced antigen-specific humoral IgG responses. Polyclonal anti-EspA antibodies were able to inhibit EHEC-induced actin rearrangements in vitro (Luan et al. 2010). Orally inoculated *S. enterica* serovar *Typhimurium* expressing intimin was able to colonize the mice Peyer's patches and spleen, producing specific serum IgG and fecal IgA antibodies and reducing EHEC shedding after challenge (Oliveira et al. 2012). In mice inoculated with EspB-expressing *L. lactis*, the antibody response consisted of not only IgG but also fecal IgA (Ahmed et al. 2013a). Recently, the immunogenicity of intranasal mice administration of a novel bivalent EHEC O157:H7 subunit vaccine,

made by antigen EspA-Tir-M, resulted in protection against EHEC O157:H7 colonization and infection at a rate of 90%. In contrast, subcutaneous immunization elicited a weak immune response and exhibited a low protection rate (Lin et al. 2017).

2.2.4 Vaccines Against Other Intestinal Pathotypes

Intestinal pathotypes differ in virulence factors and in their ability to cause a broad spectrum of diseases by different mechanisms. The enteropathogenic EPEC is defined as *E. coli* that produce a characteristic histopathology known as attaching and effacing (A/E) lesions on intestinal cells and that do not produce Shiga, Shiga-like, verocytotoxins. Typical EPEC (tEPEC) of human origin possess a virulence plasmid known as the EAF (EPEC adherence factor) plasmid that encodes localized adherence on cultured epithelial cells mediated by the bundle forming pilus (BFP), while atypical EPEC (aEPEC) does not possess this plasmid (Donnenberg and Finlay 2013). Animal pathogens and their corresponding native hosts, such as *Citrobacter rodentium* in mice and rabbit enteropathogenic *E. coli* (REPEC) in rabbits, have been widely used as model systems for EPEC infection studies. EPEC intimin has shown protection in the animal model, as immunized rabbits exhibited reduced fecal bacterial shedding, milder diarrheal symptoms, lower weight loss, and reduced colonization of REPEC in the cecum (Keller et al. 2010). In addition, an immunodominant domain of EPEC beta-intimin was protective in the REPEC challenge model (Ahmed et al. 2013b). Very recently, the immunogenic Dispersin virulence factor of EAEC, responsible for antiaggregation and bacterial penetration across the intestinal epithelium, has been proposed as vaccine antigen against EAEC infection (Asadi Karam et al. 2017).

A killed whole-cell vaccine based on a mixture of ETEC, EHEC, EIEC, EAEC, and EPEC diarrheagenic pathotypes combined with the cholera toxin B subunit (CT-B) has been proposed as vaccine inducing humoral immune response and providing protection in a mouse model following systemic or oral bacterial challenges (Gohar et al. 2016).

3 Vaccines Against *E. coli* Infections in Animals

The increasing incidence of *E. coli* foodborne-disease outbreak (FBDO) worldwide raises the urgent need for additional intervention strategies to reduce the rate of *E. coli* spreading. Identifying the major sources of risk is key to designing effective control strategies. On the other hand, a valid alternative strategy to control *E. coli* dissemination, often resulting in serious sequelae that include fatality, may consist in targeting the main animal reservoir and the primary means of human contamination. Usually, the *E. coli* transmission pathway for virulent clones to disseminate globally often has a relationship with animals and agriculture. Animal pathogenic

E. coli mainly belong to the ETEC and EPEC diarrhoeagenic, Shigatoxin-producing, uropathogenic, septicemic *E. coli* (SePEC) pathotypes as well as the avian pathogenic *E. coli* (APEC) and the mammary pathogenic *E. coli* (MPEC). Since cattle are the most important reservoir of foodborne EHEC pathogen and the root of contamination, reducing *E. coli* O157:H7 at the farm level should decrease the risk of human infection. Prevention of the *E. coli* O157:H7 global pathogen in cattle could help tackle the main reservoir of virulence, predicting a 60% decrease in human cases associated with O157:H7 assuming a bovine vaccination effectiveness of 80% and an adoption rate of 100% (Matthews et al. 2013). The ST131 pandemic in humans and other multidrug-resistant and urovirulent *E. coli* strains were found also in companion animals, ruminants, in wastewater treatment plant effluent (Johnson et al. 2009; Amos et al. 2014). Overall, *E. coli* outbreaks in humans often occurred worldwide as a result of consuming contaminated food and water, mishandling, and/or undercooking of meats or contaminated vegetables (Sharapov et al. 2016; Honish et al. 2017). Commercial vaccines against *E. coli* O157:H7 have targeted TTSS-secreted proteins (Econiche®, designed to reduce cattle contamination by EHEC), a siderophore receptor and porin proteins (SRPs) (Epitopix®, licensed for use in beef cattle in the USA). Recombinant type III secretion system (T3SS)-associated proteins EspA, intimin, and Tir from EHEC O157:H7 were proposed for calves vaccination, resulting in a reduction in EHEC shedding and in the generation of antibodies potentially cross-protective against different EHEC serotypes (McNeilly et al. 2015). Reverse vaccinology also exploited available animal-source ETEC genomes as an effective approach toward the development of subunit vaccines for animals (Dubreuil et al. 2016). Commercial vaccines for cows contain killed *E. coli* F5 isolates and/or the F5 adhesin, while commercial vaccines for female pigs contain F4 (also designated K88), F5 (K99), F6 (987P), and/or F41 fimbriae, either purified or as inactivated bacteria expressing these fimbriae with or without the LT toxoid. ETEC porcine post-weaning diarrhea (PWD) is still causing significant economic losses to swine producers worldwide. In this respect, several maternal vaccines are available. However, at weaning, lactogenic protection disappears and vaccines to protect weaned pigs from diarrhea caused by ETEC are still needed (Takeyama et al. 2015; Srivastava et al. 2016). The commercially available modified-live *E. coli* Poulvac® vaccine help protect against both the colibacillosis and productivity loss in poultry. The *E. coli* mastitis vaccine, Enviracor J-5, provides a safe and effective way to control clinical mastitis.

The major goals of veterinary vaccines are to improve the health and welfare of companion animals, increase production of livestock in a cost-effective manner, and prevent animal-to-human transmission from both domestic animals and wildlife. Interventions that would prevent zoonotic pathogens in animals will reduce *E. coli* transmission reducing the risk of contamination and bacterial spreading.

4 Adjuvants, Delivery, and Route of Immunization

Mucosal pathogens would probably require intestinal immunity, and therefore the oral route would be the preferred one for vaccine administration. Oral delivery is expected to mimic the course of natural infection that is known to confer immunity against many diarrheal *E. coli* strains. However, only a few mucosal vaccines for oral administration have been licensed for human use (Czerkinsky and Holmgren 2010). Since *E. coli* have a mucosal portal of entry and infections are confined to the mucosal surfaces, an *E. coli* vaccine should be able to induce a specific secretory IgA antibody response at the intestinal mucosa level. However, the immune response is complex and may require combinations of several immune effectors, as protection against *E. coli* does not directly correlate with mucosal sIgA content in stool or intestinal washes. On the other side, prevention against bacteremia is likely to rely on circulating antibodies capable of binding to O-antigen and promoting opsonophagocytosis. Mechanisms of protection against UTI are less well understood and may differ, such as for simple uncomplicated UTI versus persistent or recurrent UTI or for UTI in individuals with indwelling catheters. It is not known whether protection of the urinary tract would be conveyed through vaccine-induced IgA or IgG or whether urinary tract antibody levels are crucial. Thus, it is conceivable that high serum antibody levels that transudate into the mucosal tissues may be needed to achieve protection against recurrent or complicated UTI (Poolman and Wacker 2016).

Many alternative routes of vaccine delivery are being explored. In this respect, non-oral routes of immunization, such as intranasal, intradermal, sublingual, and intramuscular, are becoming very attractive because of their potential to induce a systemic and mucosal immune response. Sublingual as well as transdermal routes of administration have been shown to induce a broadly disseminated mucosal and systemic immune responses.

However, a parenteral vaccine can also elicit a mucosal immune response in individuals who have been already primed through natural mucosal exposure to the pathogen. Although IgA is the main isotype in the mucosal secretions, IgGs are also present and may contribute to the adaptive immune defenses in the gut. IgG reaches luminal secretions mainly by transudation of systemic antibodies, although small amounts are also synthesized locally. Parenteral vaccination may in itself be useful for immunization against those mucosal infections in which the pathogen is taken up or penetrates across the epithelium. In addition, parenteral administration might be used in tandem with mucosal vaccines, whether the latter are given by oral, nasal, or sublingual route. Traditional oral immunization is able to induce a substantial antibody response in the small intestine and in the ascending colon after oral immunization. On the other hand, when an *E. coli* infection occurs in the uroepithelial mucosae, which is more permeable than the intestines to transudation by plasma antibodies, a parenteral route of vaccination may also be very effective (Czerkinsky and Holmgren 2010).

Understanding cross-talk between mucosal and systemic immunity should expedite the development of vaccines against diseases caused *E. coli*. However, the gut immune system can change depending on the dietary conditions, environmental antigens, exposure to pathogens, and microbiome composition.

Cholera toxin (CT) and heat-labile enterotoxin (LT) are known to be powerful adjuvants. CT has been shown to induce increased permeability of the intestinal epithelium leading to enhanced uptake of coadministered antigens and enhanced antigen presentation by various APCs. CT markedly promoted isotype differentiation in B cells leading to increased IgA formation and exert complex stimulatory and inhibitory effects on T-cell proliferation and cytokine production. Cholera toxin and heat-labile enterotoxin (LT) also evoked both systemic and intestinal antibody responses when coadministered with protein antigens by transcutaneous immunization. Genetically detoxified derivatives of LT and CT, nontoxic but retaining the immunological and adjuvant properties of the wild-type toxins are considered the most promising adjuvants to augment local and systemic immune responses to coadministered antigens.

Vaccines against *E. coli* should be safe, immunogenic, and provide high level of protection against diarrhea in the primary target populations of infants and young children in developing countries (0–5-year-age range), and travelers to endemic areas. Moreover, an *E. coli* vaccine should be immunogenic and protective also in elderly, who are at highest risk of ExPEC bacteremia.

5 Impact of Vaccines on Microbioma

The gut microbial community (microbiota) undergoes to evolution and mutual adaptation to the host. In the postnatal period, the germ-free neonate moves from the sterile environment of its mother's uterus into a gradual colonization of mucosal and skin surfaces. During the early postnatal period, the intestinal microbiota plays a crucial role in the development of both local and systemic immunity. Then, in the intestinal tract, cholic acid, radial oxygen gradient, and dietary components become the driving forces of microbiota assembly, composition, modulation, and activities. Alterations of the normal colonization process, such as the presence of pathogenic microorganisms and toxins, can affect the important symbiotic relationship that is necessary for immune homeostasis (Walker 2013; Wu and Wu 2012). Bacteria utilize cooperative pathways to help maintaining their niches and consequently the microbial group behavior is essential to host homeostasis. These microbial relationships can be antagonistic or mutualistic, depending on the nature of the species. Bacteria express highly potent bacteriocins, microcins, and colicins to fend off other species or pathogens invading their niche without causing collateral damage to eukaryotic cells (Ohland and Macnaughton 2010). Even if the majority of genes (99.1%) examined by metagenomic sequencing of the intestinal tract consists of bacterial origin (Qin et al. 2010), also viruses, fungi, and archaea are present and may influence both specific host response and intestinal homeostasis (Norman et al.

2014). Analysis of the human microbiome indicates that proteobacteria, including *E. coli*, represent less than 0.1% of the human flora overall (Eckburg et al. 2005). Thus, vaccination against a low number of serotypes (of >180) would be unlikely to have any substantial impact on gastrointestinal and/or urogenital flora or result in serotype replacement. Emerging studies deciphering the relationship between microbiome changes and immune responses will provide more insights into the impact of the gut microbiota on vaccine efficacy (Nguyen et al. 2016). On the other hand, the interest in maintaining a healthy microbiota in commensal bacterial species with remarkable protective effects is increasing. Probiotics represent the great promise for rebuilding microbiotas and restoring health (Gensollen et al. 2016). Recent studies have addressed the issue of the potential impact of using subunit vaccines consisting of antigens that are also encoded by commensal organisms. These studies investigated the effect of vaccination with *E. coli* antigens (MipA, Skp, and ETEC_2479) conserved also in the commensals, on the intestinal mouse microbiome. Interestingly, immunization did not cause any changes to mouse health, to mouse weight gain as a function of time, or to the diversity or richness of mouse intestinal microbiomes (Hays et al. 2016).

6 Conclusions

The vaccinology field is evolving very rapidly, and new technologies are today available to make the development of effective vaccines against *E. coli* feasible in the near future. A multitude of colonization factors, toxins, and virulence determinants are necessary to allow adaptation of *E. coli* to the different niches. The enormous amount of genomic, proteomic, and transcriptomic data and their analysis could guide the search for the ideal vaccine antigens, not shared with commensal strains and with limited antigenic diversity. One approach could be to target antigens encoded by the core genome that, being shared by different pathotypes, could be potentially more cross-protective. On the other hand, including accessory antigens may be important to prevent the emergence of new pathogenic lineages. In this perspective, the subtractive reverse vaccinology approach applied to *E. coli* has allowed the identification of very promising protective antigens conserved in phylogenetically and epidemiologically distinct *E. coli* pathotypes, and has opened the way toward a universal *E. coli* vaccine.

There are still many scientific questions that need to be addressed and that could effectively guide vaccine development, such as the identification of reliable and predictive animal models, the definition of correlates of protection, the definition of relative contribution of mucosal and systemic immune response in protection, and the influence of impact of vaccination on the host microbiome. Finally, science-based studies aimed to discover the role played by any new potential vaccine antigen in virulence and pathogenesis might have a huge impact on the evaluation of the ability of the antibodies induced by such antigens in neutralizing important bacterial functional activities.

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