Non-typhoidal Salmonellosis

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Contents

Abstract

Salmonella is a ubiquitous Gram-negative bacterium belonging to the *Enterobacteriaceae* family that can infect animals and humans, with gastroenteric and systemic symptomatology from moderate to severe. Animals can act as asymptomatic carriers that excrete *Salmonella* spp. intermittently in faeces and contaminate carcasses. At present, poultry and swine are recognised as the main sources of infection for humans. Control of human salmonellosis is based on sustainable biosafety and hygienic measures "*from farm to folk*" but efficient vaccines would contribute to avoid animal infections. Since no commercial vaccines are available, a wide variety of experimental work is carried out to test both non-living and live attenuated vaccines in animal models, using either subcellular components of *Salmonella* administered with adjuvants or live genetically modified bacteria lacking structural elements, essential metabolites or virulence genes. A special effort should be conducted to design effective vaccines antigenically tagged to allow distinguishing between infected and vaccinated animals.

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19.1 Pathogen

Salmonella is a Gram-negative, facultative anaerobic, motile, non-lactose fermenting bacterium that belongs to the *Enterobacteriaceae* family. This microorganism is frequently excreted in

M. Giese (ed.), *Molecular Vaccines Volume 1*,
DOI 10.1007/978-3-7091-1419-3_19,

animal and human faeces, being thus ubiquitous and frequently found in sewage, farm effluents and any material subjected to faecal contamination.

Taxonomic classification of *Salmonella* has been complex and controversial due to the constantly increasing number of serotypes. Nowadays, *Salmonella* is classified in two species: *S. enterica* and *S. bongori* [1], and, in turn, *S. enterica* has been subdivided into six subspecies, according to their biochemical characteristics and susceptibility to bacteriophages [2]. These six subspecies were formerly named by roman numbers and currently substituted as follows:
Subspecies I: Subspecies *enterica*
Subspecies II: Subspecies *salamae*
Subspecies IIIa: Subspecies *arizonae*
Subspecies IIIb: Subspecies *diarizonae*
Subspecies IV: Subspecies *houtenae*
Subspecies VI: Subspecies *indica*

Subspecies I strains are usually isolated from humans and warm-blooded animals, while subspecies II, IIIa, IIIb, IV and VI strains and *S. bongori* are usually isolated from cold-blooded animals and the environment (rarely from humans). These six subspecies are subdivided in 51 serogroups (named either by alphabetic and/or numeric order) and more than 2,600 serotypes [3] (Table 19.1).

Classification into serotypes is carried out applying the Kauffmann-White scheme, according to characteristics of antigens: (1) somatic (O antigens) expressed by the lipopolysaccharide (LPS) O-chain; (2) flagellar (H antigens) expressed by flagellar proteins, motility, and first and second phase antigens expression; (3) capsular (Vi antigens) [2]. Accordingly, *Salmonella* strains are named orderly by the species, subspecies and the name of the serotype (if exists) or the complete antigenic *formulae*. For instance, *Salmonella enterica* subspecies *enterica* serotype Typhimurium can be also named as *Salmonella enterica* subspecies *enterica* serotype 1,4,[5],12:i:1,2. In practice (and herein), abbreviations can be "*S.*" (italics and capitals) followed by the name of the serotype in non-italics and with the first letter in capitals, e.g. *S.* Typhimurium.

Table 19.1 Classification of *Salmonella* spp. and number of serotypes identified

Species	Subspecies	Serogroup[a]	No. of serotypes
S. enterica			2,587
	enterica (I)	A-C4, D1, D2, E1-E4, F-Z, 51–54, 57, 67	1,547
	salamae (II)	B-C2, C4, D1-D3, E1, E2, F-Z, 51–53, 55–60, 65	513
	arizonae (IIIa)	F, G, I-L, O, P, R-Z, 51, 53, 56, 59, 62, 63	100
	diarizonae (IIIb)	C1, C3, C4, F-M, O, P, R-V, X-Z, 51–53, 57–61, 63, 65	341
	houtenae (IV)	C1, F, H-L, P, R-Z, 51, 53, 57	73
	indica (VI)	C1, F, H, K, S, W, Y, Z, 59	13
S. bongori (*V*)		D1, G, H, R, V, Y, 60, 61, 66	23

[a]Nomenclature by alphabetic (A–Z) or numeric (from 51 to 67) order. Serogroups C, D, Y and E are subdivided into numbers from 1 to 4

The strains most frequently isolated in clinical cases of human salmonellosis belong to serogroups A, B, C1, C2, D and E and to serotypes *S.* Enteritidis and *S.* Typhimurium [4]. Further typing, such as phage-typing and Pulsed Field Gel Electrophoresis (PFGE), can be assessed in order to classify field strains, particularly in case of outbreaks. Other characteristics of circulating *Salmonella* strains, as the antimicrobial resistance pattern, are of clinical importance to apply effective antimicrobial therapies in patients at risk and/or with complicated disease (see below).

19.2 Disease

Salmonellosis is one of the main zoonosis worldwide distributed, being an important foodborne disease in developed countries. In fact, in the USA, non-typhoid *Salmonella enterica* infections are the first cause of acute gastroenteritis,

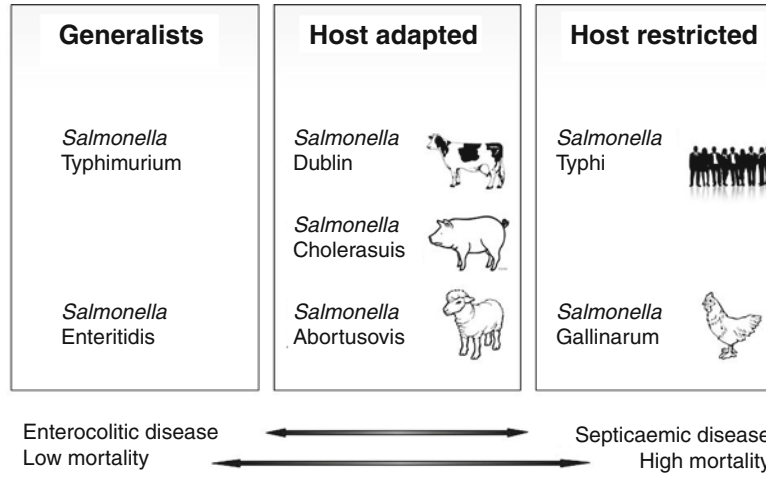

Fig. 19.1 Host adaptation in *Salmonella* infection and effects on clinical forms in the host

registering 1,027,561 human cases in 2011, from which 19,336 (1.9 %) cases required hospitalization and 378 (1.95 %) cases were lethal [5]. In the European Union (EU), salmonellosis is the second most frequent zoonosis, after campylobacteriosis, registering 99,020 human cases in 2010 [4]. During the last decade, a decreasing incidence of salmonellosis has been reported [4], probably as result of both control measures implemented in the food chain *from farm to fork* and public awareness on the importance of hygienic control measures against foodborne infections.

Among the over 2,600 serotypes of *Salmonella* species described, clinical manifestations and mortality differ according to both bacterium and host characteristics. All serotypes are considered potentially pathogenic to humans, with different degree of adaptation to the host [6] (Fig. 19.1). Thus, some serotypes such as *S*. Typhi, *S*. Paratyphi and *S*. Sendai cause severe systemic disease to humans. However, other serotypes are specifically adapted to animals, such as *S*. Choleraesuis to pigs, *S*. Dublin to cattle, *S*. Abortusovis to sheep and *S*. Gallinarum to poultry, and only occasionally affect to humans and cause only mild symptoms [7, 8]. Ubiquitous serotypes such as *S*. Typhimurium, *S*. Enteritidis and *S*. Infantis are the most recognized as zoonotic agents, affecting a wide range of animal species [9–11].

Poultry and swine asymptomatically infected, mainly by *S*. Enteritidis and *S*. Typhimurium, respectively, are considered the main sources of human *Salmonella* infections [4, 9], representing 75.6 % of total serotypes reported in human salmonellosis in the UE [6]. Other animals, such as wild birds, rodents, lizards or domestic turtles, asymptomatically infected by *Salmonella* may contribute to spread and be source of human infections [5, 12, 13].

Clinically, swine and poultry salmonellosis can produce septicaemic or enterocolitic forms (Fig. 19.1). The former is characterized by profuse diarrhoea, symptoms of systemic infection (fever, prostration, etc.) and, in absence of antimicrobial treatment, conclude with a high mortality rate. The enterocolitic form is characterized by symptoms of acute or chronic gastroenteritis, being diarrhoea the most common symptom. Animals submitted to treatment could recover from infection, eliminating the microorganism during months by faeces, for long-lasting periods, and acting as asymptomatic carriers [14]. Subclinical asymptomatic salmonellosis are the most frequent presentations in poultry and swine (the main sources of human infections), produced by a wide range of serotypes that infect tonsils, intestinal tract and mesenteric lymph nodes, and are excreted intermittently [14, 15]. While clinical forms are easily identifiable, asymptomatic carriers cannot be detected in routinely inspections, representing a major risk for humans [16].

Human salmonellosis is mainly acquired by ingestion of raw or undercooked contaminated food from animal origin, mainly from poultry

(43.8 %; eggs and meat) and pigs (26.9 %; meat), and also by ingestion of unpasteurized cow milk [11]. In fact, the decreasing incidence of human salmonellosis observed last years has been associated to the implementation of *Salmonella* control campaigns in eggs and poultry products intended for human consumption [6]. Accordingly, infections of swine origin are becoming more relevant, being *S*. Typhimurium and *S*. Derby the serotypes most common isolated in the EU [17, 18].

The human infection courses with an acute gastroenteritis that appears within 12–72 h after ingestion of contaminated food and includes symptoms as diarrhoea, vomiting, abdominal pain and fever. Treatment is based on rehydration and occasionally requires antibiotic administration, only when bacteria reach the bloodstream causing bacteraemia. This complication is particularly dangerous, even fatal, in immune-compromised patients, such as those with HIV infection, cancer or under immunosuppressive treatment, or with altering endogenous intestinal flora and, particularly, in children and aged people. In these cases, the establishment of an effective antibiotic treatment is essential to control the infection, and, thus, the antimicrobial resistance profile of the pathogen should be assessed during the earlier phase of infection. An additional problem associated to the occurrence of human salmonellosis is the emergence of *Salmonella* strains carrying resistance to multiple antimicrobial agents, frequently associated to antibiotic administration to animals.

19.3 Pathogenesis

After oral ingestion, the acidic environment of the stomach destroys a high proportion of *Salmonella*. The surviving *Salmonella* reach the distal ileum and caecum, replicate in enterocytes and go through the intestinal barrier, captured by the M cells overlying Peyer's patches, phagocytes expressing CD18 molecules and/or active entrance in non-phagocytic enterocytes (Fig. 19.2) [19]. Once in the lamina media, the invasion of the macrophages (target cells of the pathogen) requires two different type III secretion systems (T3SSs) encoded on separate *Salmonella* pathogenicity islands (SPI-1 and SPI-2) [20]. Both SPI-1 and SPI-2 provide a variety of proteins required for delivering the bacterial effectors into the host cells, modulating the host cell functions (SPI-1), such as cytoskeletal reorganization and cytokine gene expression, and the transformation of *Salmonella*-containing vacuole (SCV) into an intracellular replicative niche (SPI-2) [21]. Following the local infection of *Salmonella*, the interaction of the bacterial LPS with Toll-like

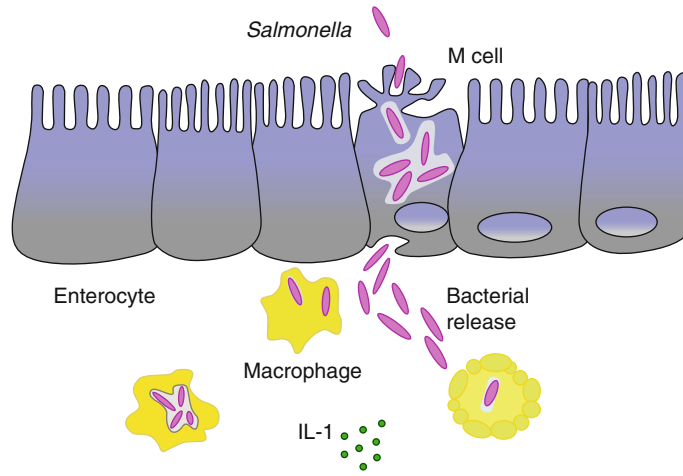

Fig. 19.2 Graphical representation of *Salmonella* pathogenesis in the intestine

receptor 4 (TLR-4) activates dendritic cells and macrophages and, subsequently, triggers the host immune response [22]. The pathogen reaches the mesenteric lymph nodes and the general lymphatic system, being disseminated through the bloodstream to spleen, liver and other organs, causing bacteraemia.

During enteric acute infection, the pathogen is massively excreted through faeces and, eventually, could induce persistent asymptomatic infections, with intermittent excretion through faeces for long-lasting periods. Asymptomatically infected hosts act as source of infection to healthy hosts, either by direct contact or indirect food contamination. Establishing persistent infections is a complex process involving both host and pathogen components.

On one hand, the host displays the innate immune response to eliminate the infection from the organisms (see below), and, on the other hand, *Salmonella* compete with host microbiota to reach its target cell and, thus, develop or activate strategies to survive in extreme environments [23]. In fact, it has been described that fimbria and adhesins are displayed as an attempt to be maintained inside the intestinal tract [24]. Similarly, resistance to stomach acid pH and ability to use inflammation related-metabolites provide a growth advantage against host microbiota [25]. Also, external factors, such as recent antibiotic treatment, could favour the establishment of *Salmonella* infection by competitive microbiota inhibition and prolong excretion of the pathogen [26]. For this reason, antibiotic therapy is only recommended for septic clinical form of *Salmonella* infections, but not for mild to moderate healthy patients (see below).

First recognition of *Salmonella* by the innate immune system is mediated by TLR-4 via MD2, as LPS specific receptor, activating the transcriptional responses to extracellular and SCV vacuolar pathogen [27]. Stimulation of this receptor triggers the expression of cytokines (such as IL-1β and TNF-α) and proteins (such as proteolytic enzymes and antimicrobial cationic peptides) from the macrophages. Thereafter, a variety of other mechanisms contribute to control *Salmonella* by the immune system, as the acidification of the SCV, defensins and reactive oxygen intermediates secretion [28]. Following primary infection, both antibodies (mainly directed to *Salmonella* LPS and proteins) and specific T-cell responses can be detected in domestic animals and humans. Humoral response is not limited to live bacteria, since non-live or subunit vaccines are able to elicit specific antibodies production, but cellular immune response is only detected after active infection. It has been widely stated elsewhere that humoral and cellular immune response levels are not always correlated with the innate immune system status to prevent or eliminate the pathogen [29].

19.4 Isolation and Typing of *Salmonella*

In order to preserve the consumer's health, EU authorities have established regulations to be accomplished compulsory in all Member States, for controlling *Salmonella* spp. in both foodstuffs and animal infections. For foodstuffs, the microbiological and hygiene criteria is established in Annex I of the Commission Regulation (EC) 2073/2005 of 15 November 2005 [30], requiring absence of *Salmonella* spp. in 5 samples of 10 or 25 g/sample, depending on the type of product, all along the product shelf life or in animal carcasses after slaughtering and before refrigeration. To control animal salmonellosis, exhaustive controls should be performed along the productive chain *from farm to fork* [31, 32]. Currently, swine salmonellosis control is being implemented in all the EU territory, after performing well-standardized reference studies in the 27 Member States [31].

The ISO 6579:2002/Am 1:2007 (ISO ahead) [33] is the standardized method internationally recommended for *Salmonella* isolation, using slight modifications depending on the type of sample (stool, lymph nodes, food, water). In general, samples are taken individually, although both animal and environmental samples could be taken in pool for epidemiological purposes. In subclinical infections, *Salmonella* might be excreted intermittently through faeces; thus, follow-up sampling should be taken in hosts nontreated with antibiotics.

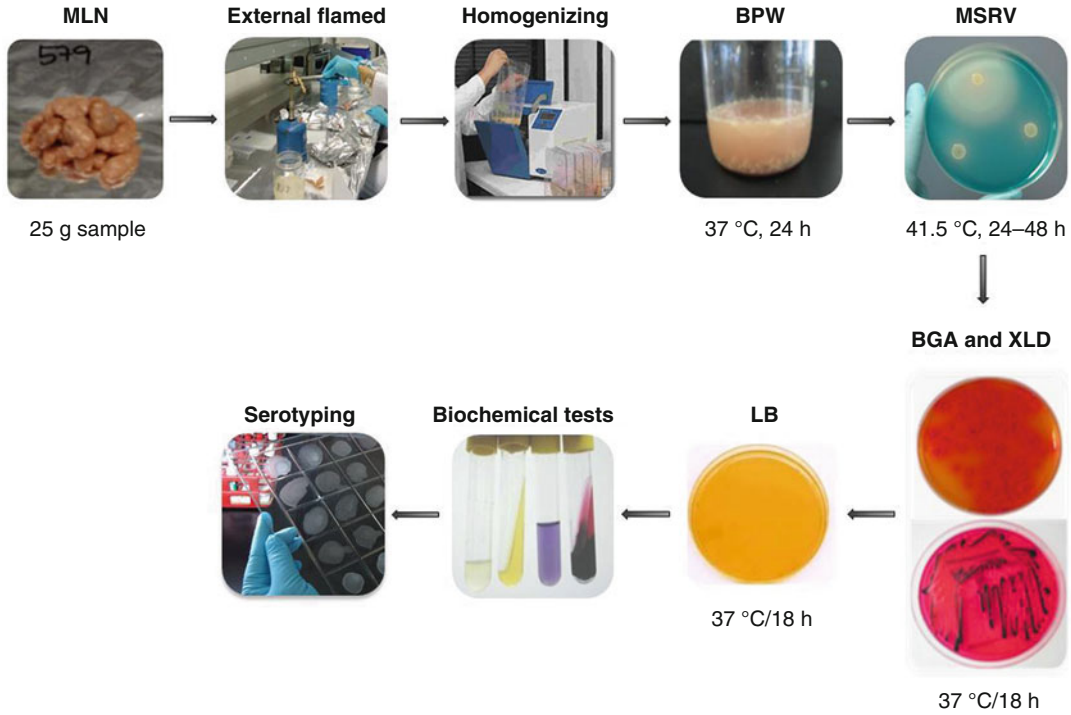

Fig. 19.3 Isolation of *Salmonella* spp. from mesenteric lymph nodes (*MLN*) following the ISO 6579:2002/Am 1:2007. *BPW* buffered peptone water, *MSRV* Modified Semisolid Rappaport-Vassiliadis medium, *BGA* Brilliant Green Agar, *XLD* Xylose Lysine Deoxycholate, *LB* Luria Bertani Agar

The ISO method comprises a stepped use of culture media, from none to highly selective, to achieve a successful isolation of *Salmonella* (Fig. 19.3). Thus, suspected colonies should be isolated after a non-selective pre-enrichment in buffered peptone water (BPW), followed by a semi-selective enrichment in Modified Semisolid Rappaport-Vassiliadis medium (MSRV) and a final selective culture in two solid selective media, such as Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA). After purification of single colonies in agar plates, biochemical tests, such as Urea agar, Lysine, Indole and Triple Sugar Iron agar (TSI) or a commercial Analytical Profile Index (API) should be applied to confirm the identity of suspected colonies. Finally, bacteria should be submitted to confirmatory serotyping by slide agglutination with specific monoclonal mouse sera directed to identify variants of the O, H and Vi antigens (see above). This technique requires the use of over 150 specific sera and carefully trained personnel, thus should be performed in *Salmonella* Reference Centres.

Additional techniques, such as phage-typing and molecular typing, can be used for a better characterization of the *Salmonella* strains, particularly useful in both global surveillance and outbreaks investigations. Phage-typing is determined by lytic or lysogenic activity of specific collections of phages, such as the 17 *S*. Enteritidis typing phages (SETP) [34] or the 34 *S*. Typhimurium typing phages [35], as described by the Health Protection Agency (HPA; Colindale, United Kingdom). Molecular typing techniques most widely used are Multiple-Locus Variable-Number Analysis (MLVA) and PFGE, the latter being the technique most widely accepted for fingerprinting strains in outbreak situations and phylogenetic studies. PFGE is relatively inexpensive but is time consuming, laborious and requires well standardization, displaying different sensitivities for different serotypes. The evolution of molecular biology has led to the emergence of novel

diagnostic techniques, such as genotyping by analysis of genes encoding O and H antigens, using multiplex PCR [36] or bead arrays [37] or ORFeome comparisons [38, 39].

19.5 Serological Diagnosis

ELISA and other serological tests can be useful tools in certain epidemiological situations [40]. However, serology is not an indicative of *Salmonella* infection at the time of sampling, since humoral response persists in the organism long-lasting periods than bacteria, and, conversely, *Salmonella* infections occur quickly, while seroconversion requires long-lasting periods.

For human diagnosis, four ELISA tests have been developed for detecting *S*. Typhi whereas are scarce for non-typhoid *Salmonella*. In fact, most laboratories currently use their own in-house tests with acceptable success, and the need for a standardized ELISA has been sidelined or, in some cases, discarded in favour of PCR or other molecular techniques. In this context, several authors point out towards the combination of LPS belonging to different serogroups, in order to improve the detection of a high number of serotypes [41].

In veterinary, a wide variety of commercial ELISA tests are available for monitoring the infection in pigs, poultry as well as food. These tests allow a quick diagnosis, using either sera or meat juice collected for animal health surveillance studies, but seroprevalence is not always in agreement with the actual infectious status, limiting the use of ELISA tests to areas with low expected *Salmonella* prevalence but not as the only infection control tool [42].

19.6 Therapy Against *Salmonella* Infection

General recommendations for enterobacterial infections treatment are hydration and soft diet, accompanied by meticulous personal hygiene. Only exceptionally, severe ill or at risk patients (immunosuppressed, infants, etc.) should be treated with antimicrobial agents. Drugs usually applied against human salmonellosis are fluoroquinolones (ciprofloxacin), trimethoprim-sulfamethoxazole or amoxicillin-clavulanic acid in adults and third-generation cephalosporin in children [43, 44]. Indiscriminate antibiotic treatment could lead to the emergence of multidrug-resistant strains, as happened after the 1980s massive treatments with ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole. In these situations, antimicrobial agents are not only noneffective in further treatments but also induce increasing illness severity and mortality rate. Moreover, it has been proven that children treated with ampicillin or amoxicillin frequently have prolonged *Salmonella* excretion periods and clinical relapses, due to the elimination of the endogenous microbiota and the strengthening of *Salmonella* throughout the gastrointestinal tract [45].

Animals are considered as the main source of multi-resistant *Salmonella* strains, as result of selective pressure derived from the systematic use of antibiotics in the diet as both growth promoters and treatment of multiple infectious processes during its productive life [46]. In 2008, in the EU, swine and cattle showed the highest number of *Salmonella* strains resistant to tetracycline, ampicillin and sulphonamides [43]. It should also be considered that the use of antimicrobials for therapy or growth promoting also disrupts the gut flora which often increases the susceptibility of pigs for *Salmonella* infection [47]. The use of antibiotics may thus act as a trigger for the spread of a *Salmonella* infection within a herd, which would not have occurred if the animals were untreated. Besides selective pressure, acquisition of antimicrobial resistance could be favoured by other factors, such as a genetic tendency of some *Salmonella* serotypes to acquire and fix genetic elements.

It can be concluded, as early also was recommended by WHO [48], that control of *Salmonella* infections should not be based on the use of antimicrobials and that the emergence of antimicrobial resistance is an additional serious reason why they should be used with great care, as exemplified by the emergence of the multi-resistant *S*. Typhimurium DT104 [49]. The

appearance of the classical profile of penta-resistance ACSSuT (aminopenicillins, chloramphenicol, streptomycin, sulphonamides and tetracyclines) in this DT104 clone has been associated to both the use of antimicrobials in animals and the international trade of infected animals [50, 51]. Thus, the emergence of *Salmonella* strains resistant to multiple antibiotics, including fluoroquinolones and third-generation cephalosporins, represents an important public health concern, recently associated to swine origin [43, 52]. In consequence, current European regulations prohibited the use of antimicrobials as growth promoters since 1 of January 2006 and recommend a limited usage of antibiotics in animals and the antimicrobial resistance surveillance of all *Salmonella* spp. isolates [53].

Otherwise, alternative novel therapies based on diets that modify the composition of endogenous intestinal bacterial flora are being implemented to favour microorganisms that contribute to eliminate *Salmonella* from the gastrointestinal tract [54].

19.7 Vaccine Working Mechanisms

Seroconversion, based on IgM followed by IgG antibodies, is induced at the first stage after vaccination. Moreover, IgA production can be detected in case of mucosal vaccination. LPS, flagellin, fimbriae and other proteins (lipoproteins, outer membrane proteins, heat shock proteins) are the main responsible elements for the stimulation of the immune system. The elicitation of the humoral immune response has been cited in a wide range of hosts, from laboratory experimental models (mice) to livestock (i.e. calves) and humans, receiving subunit, killed or live attenuated vaccines. Taking into account all the results described in the literature, a correlation between the presence of antibodies and resistance to infection cannot always be established. Moreover, protective effects based on the humoral immune response have only been described in some experimental conditions such as low dosage or moderately virulent strains challenge.

Live attenuated, but not subunit and killed, vaccines have been described as capable of inducing Th1 cellular immune responses (CD4 and CD8 lymphocytes), accompanied by the presence of cytokines (IL-12, IFN-γ) in different hosts. In fact, some subunit and killed vaccines have been reported to be Th2 immune response inductors. As it has been stated above regarding the humoral response, cellular immune response does not always correlate with protection after administration of *Salmonella* vaccines. Therefore, further investigations are needed to the elucidation of the mechanisms of protective immunity against *Salmonella*.

19.8 Vaccine Control and Animal Models

The approach to ensuring the purity, safety, potency and efficacy of veterinary vaccines may vary from country to country depending on local needs. However, proper standards and production controls are essential to ensure the availability of consistent, high-quality products for use in animal health programmes [55]. The designed vaccine should be tested in terms of purity (Gram staining, homogeneity of growth in culture media and sero- and phage-typing), innocuousness (lethality in mice and the final host, side effects, stability and absence of reversion in case of live attenuated vaccines, transmission to milk or eggs), efficacy (level of protection in both mice and final host) and environmental behaviour (persistence of the vaccine in stools and litter, capability of infecting surrounding animals) [55]. Besides, all these final control measurements should be applied during the production of different batches, to ensure homogeneity in the method of manufacturing.

Primate salmonellosis closely resembles human symptomatology but the use of these animals is very limited due to practical, economic and ethical reasons. Otherwise, mice and calf models have been successfully used for elucidating both virulence and immunological mechanisms [56, 57]. Mice experimental infection with *Salmonella* causes rapid systemic infection, evidenced by symptoms as fever,

piloerection, prostration and starvation, followed by liver and spleen colonization and finally death [58]. Moreover, mice have been used to evaluate the efficacy of *Salmonella* vaccines either against lethal [59] or sublethal [60, 61] challenges.

Thus, human enteritis is characterized by diarrhoea, the bovine model has been considered suitable, since calves infected by *S*. Dublin displayed bacteraemia, abortions and became as chronic carriers, whereas calves receiving *S*. Typhimurium were asymptomatic carriers [58]. The model of ligation iliac loop, in calves, pigs, rabbits and mice, has been widely used due to its potential to study bacterial virulence factors and early gastrointestinal steps of infection, although this model does not predict the degree of protection of a vaccine [58].

19.9 Vaccines and Rationale for Vaccination

As zoonotic agent, prevalence of *Salmonella* infections in humans is directly related to prevalence in animals. Since there are no anti-*Salmonella* vaccines safe enough to be applied in humans, the control of this infection should be based on animal prophylaxis and hygienic measures directed to avoid dissemination of the pathogen to both other animals and humans, the latter mainly through foodborne of poultry and swine origin. In order to prevent the consumer's health, current legislations involve a complete control of *Salmonella* "*from farm to fork*" [31, 32]. Critical points throughout primary production, e.g. feed testing, health and hygienic controls at slaughter and hygienic measures during handling and consumption of poultry and swine meat and derivatives, are considered essential to control *Salmonella* dissemination and infections.

19.9.1 Nonliving Vaccines

Inactivated and subunit vaccines have been used widely in the past, in both humans and animals, with variable success [62, 63]. Different simple or combined bacterial fractions, such as Vi-polysaccharide, LPS, O-Chain, fimbriae or porins, have been used as non-live vaccines. For instance, combination of LPS to protein carriers has demonstrated to induce antibody responses in rabbits and mice but limited efficacy against a challenge infection [64]. In order to improve the efficacy, non-live vaccines require to be administered in combination with classical (e.g. aluminium hydroxide, complete (CFA) and incomplete (IFA) Freund's adjuvants) or novel (e.g. extra domain A of fibronectin (EDA) [61]) adjuvants. In general, non-live vaccines are safe but induce strong humoral responses and poor Th1 cell-mediated immunity, leading to low efficacy [29, 65] and undesirable serological interference with the diagnosis of the infection, being LPS and fimbriae the immunodominant antigens of *Salmonella* used in diagnostic tests. In fact, differentiation of infected and vaccinated animals (DIVA) is a priority in the design of vaccines against animal salmonellosis (see below).

Current investigations in animal salmonellosis vaccines are mainly directed towards the design of live vaccines allowing an attenuated, safe and efficient *Salmonella* strain that, in turn, induces a serological response allowing DIVA. However, the concept of attenuation varies depending on both the *Salmonella* serotype and the animal species involved since, in fact, most of *Salmonella* serotypes affecting humans are "attenuated" for animals, without inducing illness (see above). Accordingly, a "safe" *Salmonella* vaccine should be understood as unable to be excreted and contaminate both environment and food chain.

19.9.2 Live Attenuated Vaccines

In general, live vaccines are considered better than inactivated vaccines, since the former could (1) induce both cell-mediated and humoral immune responses, (2) be effective after one single-dose administration, (3) induce mucosal immune response, after oral administration, (4) be used as carrier for delivery of other recombinant antigens and become a multivalent vaccine and (5) have low cost of production and easy storage [29].

Early developed live *Salmonella* vaccines were spontaneous mutants obtained after *in vitro* culture, as *S*. Gallinarum 9R [66]; temperature treatment, as TS *S*. Typhi [67]; chemical selective pressure, as nitrosoguanidine in *S*. Typhimurium-NTG [68] or streptomycin in *S*. Abortusovis Rv6 [69]; or ultraviolet radiation [70]. These vaccines have proven to be effective in mice, poultry and cattle, and some of them have been licensed for their use. However, severe side effects, such as septic arthritis or hepatitis, have been described [71]. Advances in both *Salmonella* pathogenomic knowledge and molecular biology technology have open the possibility to design new live *Salmonella* attenuated strains with well-defined and non-reverting mutations in genes related to virulence and/or immunogenicity. The functional identification of *Salmonella* genes has led the possibility to select and mutate those involved in the *in vivo* bacterium survival and infection processes, including those encoding bacterial structural components and essential metabolite biosynthesis and virulence genes. All of them are described below.

19.9.2.1 Mutants in Bacterial Structural Components

Since LPS is both a major virulence factor and the immunodominant antigen in serological diagnostic tests, development of rough LPS mutants has been an interesting approach to build vaccines allowing DIVA. In general, complete LPS core *Salmonella* mutants are considered more effective than deep rough mutants against a virulent infection. In fact, several *Salmonella* mutants lacking different LPS portions, such as Δ*waaH* and Δ*waaL*, have been proposed as live vaccine candidates [61, 72, 73], but other rough mutants have been considered too attenuated (e.g. *S*. Typhimurium Δ*waaG*) to confer protection enough to prevent virulent infections or too virulent to be safe vaccines, being discarded as vaccine candidates [74].

Since galactose is a component of the LPS core, galactose epimeraseless or *galE* mutants are unable to synthetize the enzyme uridine diphosphate galactose (UDP-Gal) epimerase and, therefore, do not convert the uridine diphosphate glucose (UDP-Glu) to UDP-Gal and vice versa. This type of Δ*galE* mutants was developed in eighties, showing an incomplete LPS (deep rough phenotype) in absence of this sugar *in vitro*. However, if galactose is exogenously provided, like *in vivo*, Δ*galE* mutants could revert to smooth phenotype and, therefore, revert to virulent form. This phenomenon, which has been described in calves vaccinated with *S*. Typhimurium Δ*galE* mutant, could generate not only non-protection against a virulent challenge but also induce diarrhoea, fever and even death in calves [75, 76]. Similar results were obtained with Δ*galE* mutants in *S*. Typhi [77] and *S*. Enteritidis [78] genetic backgrounds. In an attempt to avoid rough-to-smooth phenotype reversion, a *S*. Enteritidis Δ*gal* operon (including *galM*, *galK*, *galT* and *galE* genes) mutant has been described [61]. Despite absence of side effects, no protection was observed in mice vaccinated with *S*. Enteritidis Δ*gal* operon and challenged by intraperitoneal route, indicating that deep rough mutants are not effective vaccines against smooth *Salmonella* infection [61].

Synthesis of outer membrane proteins (Omp) OmpC and OmpF is regulated by *ompR* gene. *S*. Enteritidis Δ*ompR* gene was highly attenuated and able to induce a moderate protection after oral challenge [79]. Besides, individual Δ*ompC* and Δ*ompF* attenuated mutants have been described. In the DIVA context, Omp mutants arise as an alternative to LPS mutants leading to absence of anti-Omp antibodies in vaccinated but not infected animals.

19.9.2.2 Mutants in Bacterial Essential Metabolites

Mutations in genes encoding the aromatic (*aro*) synthetic pathway have been described as attenuated and effective vaccines in different animal models [29]. Genes *aroA*, *aroC* and *aroD* have been widely used to design single and double mutant vaccines, in *S*. Dublin and *S*. Typhimurium for calves [80] and *S*. Enteritidis and *S*. Gallinarum for chickens [81]. Moreover, Δ*aroC*Δ*aroD* and Δ*aroA*Δ*aroC* double mutants maintain the immunogenicity with minimal chance to revert to virulent phenotype, being thus proposed as candidate vaccines [82].

Genes blocking the synthesis of adenosine monophosphate (*pur* mutants) require an external input for adenine, leading to a drastic *in vivo* attenuation. *S*. Typhimurium and *S*. Dublin Δ*purA* mutants demonstrated a reduced ability to colonise and persist in mice, stimulating an insufficient immune response and leading to low protection level [83, 84].

Finally, adenylate cyclase (*cya*) and cyclic AMP receptor (*crp*) genes regulate the expression of other genes involved in the utilization of carbohydrates, amino acids and cell surface structures, such as Omps, fimbriae and flagella. Despite Δ*cya* and Δ*crp* mutants are highly attenuated and their survival in the spleen is very limited, oral immunization has led to protection in mice against an oral challenge. The *S*. Typhimurium and *S*. Cholerasuis Δ*cya*Δ*crpA* double mutants were effective against parental or oral challenges, in chickens and swine, respectively [85, 86].

19.9.2.3 Mutants in Bacterial Virulence Genes

Salmonella virulence genes have been studied in order to reduce its capability of growing in the host but maintaining the stimulation of the host immune system to fight against virulent infections. In this context, several genes, both chromosomic (*invA*, *hilA*, *PhoP*/*PhoQ* two-component regulatory system) and plasmidic (*spvB*, *spvC*), have displayed different degrees of virulence. For instance, whereas *S*. Enteritidis Δ*hilA*, Δ*spvB* and Δ*spvC* mutants showed moderate to high virulence, Δ*invA* and Δ*phoP* mutants showed low virulence but also low protection against a virulent infection [78, 87].

Conclusions

Salmonellosis is a major zoonosis that is mainly acquired through food. Incidence of human salmonellosis is directly related to incidence of infection in poultry and swine, frequently asymptomatic. Since there are no vaccines safe enough to be administered to humans, health authorities advise a control of the infection at the animal stage "from farm to fork". Since antimicrobial treatments should be avoided at farm level in order to avoid the emergence of *Salmonella* strains with multidrug resistance, the animal vaccination could successfully reinforce (but not to substitute) the control programmes based on hygienic and sanitary measures. Live attenuated vaccines are generally more effective than subcellular vaccines. However, live vaccines retain some residual virulence, and its large-scale application may involve a risk of introducing new pathogens genetically modified into the food chain. Since a practical point of view, future directions in developing animal vaccines should be focused on new *Salmonella* live vaccines able to allow discriminating between infected and vaccinated animals (DIVA).

Acknowledgements Contract of B. San Román was financed by CSIC (Programme JAE-Doc) and that of V. Garrido by Gobierno de Navarra (project IIQ14064.RI1).

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