

## Salmonella vaccines

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*Salmonella* is one of the most prevalent food-borne diseases over the world. Reducing the incidence of *Salmonella* at farm level will lower its incidence through the rest of the food chain. Both the World Health Organisation (WHO) and the European Union have laid down guidelines to eradicate this pandemic, including the mandatory vaccination from 2008 onwards for laying hens in those European countries with a prevalence above 10%. What sort of vaccines is available? What should we expect from these measures?. Evolution, epidemiology, pathogenesis and safety are some main pillars under the proposed massive vaccination against salmonellosis.

**Keywords:** *Salmonella*, vaccine, adjuvant, pathogenicity, virulence

### 1. *Salmonella*, the diversity.

*Salmonellae* are divided taxonomically into two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is comprised of more than 2,500 serovars which is clustered into seven subspecies designated I, II, IIIa, IIIb, IV, VI, and VII. Subspecies I contains 99% of human-pathogenic serovars, including Typhi, a human-adapted serotype that is the causal agent of typhoid fever, and Enteritidis, which is responsible for gastroenteritis in humans. Due to the clinical importance of some of these serovars, the current nomenclature accepts the omission of the species in their nomination. For example, *Salmonella enterica* subspecies *enterica* serotype Enteritidis can be referred to simply as *Salmonella* Enteritidis, nomenclature that will be used along this chapter.

There is a great diversity in this genus, since we are dealing with an old pathogen that probably evolved from reptiles and adapted to mammals 200 hundred millions years ago. In fact, currently, *Salmonella* serovars may be able to infect a wide phylogenetic range of hosts, from reptiles and birds to mammals, including humans. This is a main inconvenient to control the spread of any infection through vaccination. It is well recognized that multiple potential hosts and reservoirs are not compatible with the success of a vaccine: which species should be vaccinated? what about the wildlife animals or even pets? For instance, the intestinal carriage of *Salmonella* by healthy dogs and cats is very common. Between 3% and 5% of all cases of salmonellosis in humans have been associated with exposure to exotic pets, mainly reptiles (turtles, snakes, iguanas, lizards, etc.), and as high as 90% of reptiles may be *Salmonella* carriers [1].

The serovar *Salmonella* Enteritidis remains one of the main causes of food-borne illness and as such is considered to be the most important pandemic zoonosis produced under natural conditions [2, 3]. It is estimated that the worldwide incidence is more than a thousand million cases and results in three million deaths per year. In the United States alone, *S. Enteritidis* has emerged as the major etiologic agent of human salmonellosis resulting in 2–4 million cases yearly. Domesticated fowl and their products are recognised as the most prevalent source of infection of *Salmonella* Enteritidis in humans [3]. Consequently, the European Commission has proposed that the vaccination of laying hens may be the most practical measure to control the disease in humans. However, present vaccines have limited efficacy, hence,

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the development of stable, efficacious, and safe vaccines against *S. Enteritidis* is still a major challenge [4, 5].

## 2. Vaccination: Immunity and Protection

Protection and immunity are analogous terms for the same meaning: freedom from the pathogen. The cardinal property for a pathogen is to colonize and invade the body (only a very small number of pathogens do not invade, causing diseases by secreting exotoxins), and the term immunity reflects the condition of being able to resist a particular disease, especially through preventing the development of a pathogenic microorganism or by counteracting the effects of its products [lat. *immunitas*, freedom from]. Thus, the ultimate goal of a vaccine is to develop long-lived immunological protection, whereby the first encounter with a pathogen is 'remembered' by the immune system, and as a result, the infection is either completely prevented or the severity of the disease greatly reduced.

In birds, the **oral infection** with the broad-host-range serovars *S. Enteritidis* is characterised by a bacterial translocation through the intestinal epithelial barrier, where the majority of the infecting inoculum is rapidly cleared. Only a small number of microorganisms successfully access the mononuclear phagocyte system, in which they either multiply exponentially or induce apoptosis leading to inflammation via pro-inflammatory cytokines. Impaired bacterial replication within the MPS results in a plateau phase, during which IFN $\gamma$  and TNF $\alpha$  stimulate macrophage and NK cell-dependent effector mechanisms. Finally, specific T-cell-dependent immunity achieves clearance of the bacteria from infected organs.

On the other hand, bacterial colonisation is established within the two caeca, which represent a reservoir of infection of *Salmonella* in poultry [6], where persist for months without triggering clinical signs, except on very young chicks, in which high mortality rates are recorded. *Salmonella* may be shed in the feces and can lead to horizontal transmission to other birds in the flock as well as to contamination of the meat after slaughtering.

Nowadays, the importance of vaccination in the control of infectious diseases is unquestionable; the progress that has been made in human and animal health during the last few decades have guaranteed that. Attending to their intrinsic nature, they can be sorted in three main categories: attenuated, bacterins, and subunit vaccines (Table 1). However, when it comes to valuing the cost/benefits of a vaccine, we need to take into account not only that the vaccine prevents the illness, but in addition, it should avoid leading to negative effects for the immunized animal or for the handler. Live attenuated vaccines are still by far the most utilized for their efficiency with respect inactivated (bacterins) and acellular ones, but is it necessary to run the risk of using an alive vaccinal strain?

### 2.1. Live attenuated vaccines

Live attenuated vaccines have multiple advantages over nonviable vaccines because of their ease of administration, ability to carry heterologous antigens, and capacity to induce mucosal, cellular, and humoral immune responses. The aim of attenuation is to diminish the virulence of the pathogen, but retaining its immunogenicity. Since the methods of empirical attenuation developed by Pasteur and Koch at the end of the twentieth century, many successful live viral and bacterial vaccines are produced by repetitive *in vitro* passage in cell culture or by specific mutagenesis. The knowledge we have nowadays about genomics allows us to selectively knock out concrete virulence genes. Advantages of this strategy are that some important antigenic determinants can be retained by attenuated strains, able to elicit both antibody and cellular immunity. Besides, the growing capacity of these attenuated vaccines provides prolonged exposure of antigens to the immune system, resulting in the production of long-lasting memory cells. Attenuated mutations fall into two general categories: metabolic functions or virulence factors. The most widely studied metabolically attenuated strains include mutants deficient in the biosynthesis of aromatic amino acids (i.e. *aroA*, or *aroC* and *aroD*) or purines (*purA*, *purE*) or in the production of adenylate cyclase (*cya*) or the cyclic AMP receptor protein (*crp*). In general, auxotrophy can interfere with

bacterial replication within the host whenever the required metabolites are absent from, or present in amounts insufficient for bacterial growth in, the compartment where the bacteria reside

The two more extensively characterised virulence-attenuated vaccine strains have mutations in the two-component regulatory system *phoP/phoQ* or in *Salmonella* pathogenicity island 2 (SPI2) loci. Live attenuated *Salmonella* vaccines offer varying degrees of protection in chickens [6-10]. Several risks, however, are associated with live vaccines, especially in immunocompromised individuals:

Some of the structural components of the microorganism used may contain immunosuppressive antigens. Attenuated strains may still have some residual virulence due to an incomplete inactivation.

Attenuated strains, after being released into the environment, can recover their virulence in other hosts, or can acquire genes from other microorganisms by natural genetic transfer.

Under these points of view, despite their extended use, the restrictions for the use of attenuated modified organisms in vaccination are becoming more and more stringent.

## 2.2. Killed whole organisms

To avoid the risk of live vaccines, the use of killed organisms was introduced as safer vaccines. These vaccines are made from the entire organism but inactivated (killed) by physical or chemical agents. However, protection induced by bacterins in poultry is generally modest [11-14]. The limitations of these kinds of vaccines are that their immunogenicity usually has to be enhanced by coadministration with adjuvants, and, in any case, multiple doses are necessary for obtaining long-term protective immunity; besides, as live vaccines, they may contain immunosuppressive antigens.

## 2.3. Subunit vaccines

Subunit vaccines containing immunodominant components of the bacteria may offer an alternative. Subunit vaccines may be crude or purified extracts of the pathogen, synthetic peptides or may be obtained by the use of recombinant DNA technology, pure DNA or RNA. The primary goal of this approach is to identify the individual antigens of the pathogen that are involved in inducing protection, avoiding the immunosuppressive ones. Combining genomics with our understanding of pathogenesis, it is possible to identify specific proteins from most pathogens that are critical in inducing the right protective immune responses. The potential advantages are their safety, the potential abilities to target the vaccines to the site where immunity is required, and to differentiate vaccinated animals from the infected ones through the right selection of the components. In spite of these data, low levels of resistance against salmonellosis can be induced by administration of flagella, porins or polysaccharide fractions [20-28]. The potency of these vaccines is often poor when administered without adjuvant and/or a delivery system. New-generation adjuvants are designed to induce minimal side effects, enhance the duration of the immune response, and concurrently stimulate humoral, cellular, and mucosal immune responses. Furthermore, an ideal adjuvant would be biodegradable, economical, and simple to manufacture

## Immunoadjuvants

Adjuvants (lat, *adjuvare*, aid) are defined as a group of structurally heterogenous compounds that enhance or modulate the immunogenicity of the associated antigens. Despite the recognition of many different types of adjuvant, however, little is known about their mode of action. Janeway [15] called adjuvants “the immunologists dirty little secret”, because their mode of action is poorly understood. The events triggered by these immunomodulators appear to come from one or the combination of several of the following effects:

**Depot effect.** It is well known that antigens in solution are mostly quickly removed by neutrophils and macrophages, but subsequently, they are unable to prime naive T cells. Therefore, following the antigen's disappearance the immune response is hardly detectable. The most used adjuvants, such as oil-emulsions and antigen-absorbing aluminium salts, may retain antigen at the injection site, from where it is released

in minute quantities over a prolonged period of time. These compounds mainly stimulate the production of antibodies by the induction of Th2-lymphocytes (Figure 1). In the case of use of alum, the mechanism of action seems to be due, at least in part, to the formation of a depot of free alum that will induce the recruitment and activation of immune cells to the site of inoculation. However, this “favourable” local inflammation may derive in a granulome, or even eosinophilia [16, 17]. Besides, these adjuvants may produce allergic reactions after a reimmunization.

**Effect on Antigen presenting cells (APC).** The adjuvant-induced enhancement of an immune response may be ascribed to the improved delivery of antigens into the draining lymph nodes. This may be achieved by facilitating the antigen uptake by APCs (Figure 1), or by increasing the influx of APCs into the injection site. Whichever is the case, the result is the same: an effective priming of specific T cells derived from an increase in the provision of antigen-loaded APCs, promoting the activation state of APCs by upregulating costimulatory signals or MHC expression. This results in the corresponding cytokine release, enhancing the speed, magnitude and duration of the specific immune response. Some vectors are able to target associated antigens into APCs, including DDS (pluronic micelles, liposomes, IS-COMs, and polymeric particles).

**Nonspecific immunostimulating effect.** Some agents can stimulate the non-specific component of the immune system. Numerous microorganisms contain “alert signals”, the so called “microbial or pathogen associated molecular patterns” (MAMPs or PAMPs, respectively), not present in mammalian cells (Figure 1). These structures activate immune cells through interaction with specific receptors (toll like receptors, TLRs). Some examples are: lipopolisaccharide (LPS), monophosphoryl lipid A (MPL), flagellin, lipoproteins, muramyl dipeptide (MDP); trehalose dimycolate (TDM), or CpG DNA, among others [18-23]. Besides, the special chemical nature of some polymers used in the formulation of vaccine delivery systems may also be recognized as scavenger ligands for the APCs [24, 25].

**Particulated carrier systems** have been proposed to improve the mucosal bioavailability of antigens allowing a single dose [26-29]. These carriers protect labile molecules from degradation in the gastrointestinal tract. Nanoparticles are submicron-sized colloidal systems that may protect antigen against chemical enzymatic or immunological degradation, and facilitates targeting and presentation of antigens to inductive sites of mucosal immune system. Their basic colloidal properties and degradation depend on copolymer composition. The adjuvant capacity of these nanoparticles (NP) towards a single soluble protein like ovalbumin and bacterial extracts from *Salmonella* spp. was previously investigated. Results were highly suggestive for the use of NPs as an efficient antigen delivery system, especially when a long lasting Th1 cytokine response is required.

**Table 1 .** General types of vaccines and their comparison attending to the ideal properties

Ideal vaccinal properties	Attenuated	Inactivated	Subunit	
			Classical Adjuvant (Alum)	New adjuvants (DDS)
I	+++	+	+	+++
II	+++	+	+	+++
III	+	++	++	+++
IV	+	+	+	+++
V	+	+++	+	+++

Current vaccines may fall in three major categories: attenuated, inactivated and subunit. The table shows a comparison of such vaccines.

Several factors must be kept in mind in developing a successful vaccine:

I) A good vaccine should stimulate a strong, protective and long lasting immune response. Through the induction of strong, long-lived immunological specific T and B cell memory cells. Measurement of the specific subsets elicited by immunization may guide vaccine development.

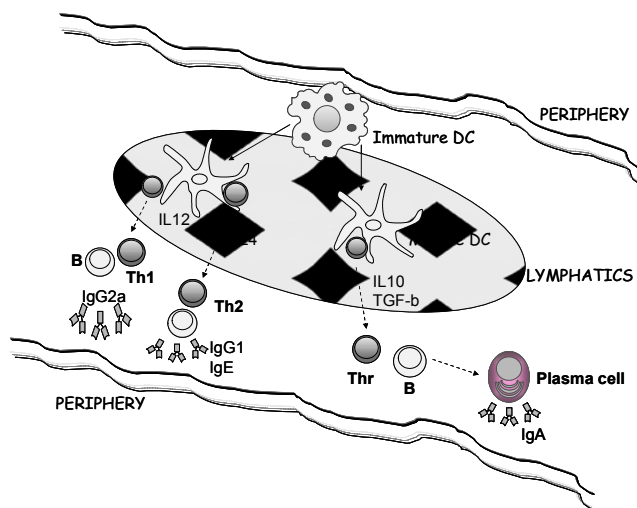
II) A good vaccine should induce the right sort of immune responses. The protective immune responses against extracellular pathogens seem to be mediated by long-lived humoral immune responses

through the production of antibodies. However, in the control of intracellular infection, cellular immune responses have been shown to be crucial in mediating protection. Therefore, the development of a successful vaccine against those diseases will be facilitated by a thorough understanding of how cellular immune responses are generated and maintained *in vivo*.

III) An ideal vaccine should be safe, including children, elderly and immunocompromised subjects. Despite the success of vaccination in eliminating disease and death, the public acceptance of even minor side effects of vaccination is very low. One major challenge faced in developing new vaccines is to achieve strong immunogenicity without increasing reactogenicity.

IV) A single dose of vaccine should confer robust, long-lived immunity. Only a few live vaccines have achieved this goal. In contrast to the results with live vaccines, it has been difficult to promote long-lived immunity with a single dose of non-living antigen vaccines. One goal of vaccine development is to rectify this using new adjuvants and antigen delivery systems.

V) An ideal vaccine should be affordable by the population at which they are aimed and should be formulated to resist high and low temperatures to facilitate distribution. This is a main problem for alive attenuated vaccines



**Figure 1.** Diagram showing the central role of Antigen presenting cells on the elicited immune response

1. Immature subsets of DC patrol peripheral tissues and they are armed to capture antigens by several mechanisms. If antigens or particles contain any MAMP on its surface, DC undergo a process called "maturation": processing and exposition to the surface of antigenic processed fragments in association with MHC I or MHC II molecules, associated with an increase in the expression of co-stimulatory molecules (B7 molecules), and, also, of chemokines expression (CCR7 upregulation). As a consequence, they migrate to the T zone of the secondary lymphatic organs.
2. Naive T cells in the secondary lymphatic organs activation require at least two signals: the complexes peptide-MHC II or peptide-MHC I, and the presence of costimulatory molecules such as B7 molecules (expressed on the surface of mature DC). If the level of stimulation is adequate, it results the clonal expansion of T lymphocytes.
3. Their differentiation towards Thelper type 1 or 2 will depend on the implied DC subset, the maturation stimulus, costimulatory molecules and the cytokines environment. The polarization of Th1 cells is a direct consequence of the capacity to activate DCs to secrete IFN $\gamma$ -promoting factors. The interaction of microbial components with pattern recognition receptors (PRRs) on DCs results in the synthesis of such Th1-promoting cytokines (IL-12, IL-18, IL-23). Naive Th precursors become susceptible to these cytokines and express the gene encoding IFN $\gamma$ . The new effector Th1 cells continue to express functional

receptors for the IFN $\gamma$ -promoting factors (IL-12, IL-18 and IL-23) licensing DC for activating CD8<sup>+</sup> T cells towards Cytotoxic T lymphocytes (CTL). On the other hand, the polarization of Th2 cells occur following a more simplistic combined signalling involving T-cell receptor, CD4, CD28, and the action of IL-4.

T-helper lymphocytes and CTL cells migrate to the source of antigen in peripheral tissues. When the antigen has been eliminated, the most of the effector cells die, but a small fraction remains as long-lived memory cells. Whereas effector memory cells home to peripheral tissues, central memory cells continue to recirculate through lymph nodes in which they mount strong recall responses whenever the antigen returns.

T-helper lymphocytes also migrate to the B cell areas stimulating the B cell expansion, isotype switching, affinity maturation. In response to antigen recall the clones of memory B cells proliferate and differentiate rapidly into antibodies secreting cells (regulated by memory T-helper cells). In rodents, cytokines secreted by Th1 lymphocytes induced IgG2a antibody isotype whereas Th2 is associated with the induction of IgG1. DC may also directly activate naive and memory B cells towards antibody producing plasma cells.

Protective immune response raised in mice against *Salmonella* has been attributed to the right balance between antibody response and cellular mediated immune response, balanced toward the Th1 subset.

### Summing up

Domesticated fowl and their products are recognised as the most prevalent source of infection of *Salmonella* Enteritidis in humans. Both the World Health organisation (WHO) and the European Union have laid down guidelines to eradicate this pandemic. Vaccination is generally accepted as the most practical measure in that is easy to apply and the most economic, however, present vaccines have limited efficacy. Therefore, the development of stable, efficacious, and safe vaccines to prevent human and poultry salmonellosis is a major challenge. Live attenuated vaccines are still, by far, the most utilized for their efficiency with respect to inactivated (bacterins) and subunit ones, but it remains clear that the cost/benefit ratio is in favour of the subunit vaccines. However, they have been implicated in a series of immunodeficiencies that increase susceptibility to *Salmonella* infection and maybe hazardous. Additionally, the large scale use of live vaccines must be weighed against the introduction of potential human pathogens into animals which eventually may be consumed by humans. However, as established above, these non-replicating vaccines suffer in general from immunogenicity requiring, therefore, the use of adjuvants, and this is the aim where antigen delivery systems may be applied. DDS may be designed in order to be used as adjuvants compiling the three main mechanisms of action. Furthermore, a problem that should be considered in vaccination is the interference with serodiagnosis of natural infection. Taking into account that in vaccinology the golden standard should be that one that does not interfere with serodiagnosis, and that in salmonellosis the most used diagnosis tests are based on detection of antibodies against the "O" chain of the S-LPS or/and flagellin; therefore, it is under research the use of subcellular extracts from rough strains lacking flagellin.

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