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REVIEW ARTICLE
Vaccination and early protection against non-host-specific *Salmonella* serotypes in poultry: exploitation of innate immunity and microbial activity

F. VAN IMMERSEEL[1], U. METHNER[2], I. RYCHLIK[3], B. NAGY[4], P. VELGE[5], G. MARTIN[2], N. FOSTER[6], R. DUCATELLE[1] AND P. A. BARROW[6]*

[1] *Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium*
[2] *Institut für bakterielle Infektionen und Zoonosen im Friedrich-Loeffler-Institut, Jena, Germany*
[3] *Veterinary Research Institute, Brno, Czech Republic*
[4] *Veterinary Medical Institute of the Hungarian Academy of Sciences, Budapest, Hungary*
[5] *Institut National de la Recherche Agronomique, UR 918 Pathologie Infectieuse et Immunologie, Nouzilly, France*
[6] *Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire, UK*

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SUMMARY

A recent European Union Directive required member states to put monitoring and control programmes in place, of which vaccination is a central component. Live *Salmonella* vaccines generally confer better protection than killed vaccines, because the former stimulate both cell-mediated and humoral immunity. Administering *Salmonella* bacteria orally to newly hatched chickens results in extensive gut colonization and a strong adaptive immune stimulus but broiler chickens are immunologically immature. However, colonization exerts a variety of rapid (within 24 h) protective effects. These include specific colonization-inhibition (competitive exclusion) in which the protective bacteria exert a profound resistance to establishment and colonization by other related bacteria. This is thought to be primarily a metabolic attribute of the vaccinating bacteria but may also involve competition for attachment sites. The presence of large numbers of bacteria originating from a live *Salmonella* vaccine in the intestine can also induce infiltration of polymorphonuclear cells into the intestinal wall, which confers resistance to invasion and systemic spread by virulent *Salmonella* strains. This opens new perspectives for vaccine usage in broilers, layers and breeding poultry but also in other animals which show increased susceptibility to infection because of their young age or for other reasons, such as oral chemoprophylaxis or chemotherapy, where the lack of established normal gut flora is an issue. We recommend that all live vaccines considered for oral administration should be tested for their ability to induce the two protective effects described above. Further developments in live *Salmonella* vaccines are, however, currently hindered by fears associated with the use and release of live vaccines which may be genetically modified.

INTRODUCTION

Poultry products (eggs and meat) are still thought to be the main sources of human foodborne infections caused by *Salmonella* in Western countries [1–4]. Incidents are generally sporadic, affecting individuals, but outbreaks are common and can occasionally involve large numbers of cases. Shell eggs are the most common vehicle of infection in outbreaks [5, 6]. For sporadic *Salmonella* infections, the consumption of undercooked hen eggs, egg products and poultry

* Author for correspondence: Professor P. A. Barrow, Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG20 7NN, UK.
(Email: paul.barrow@bbsrc.ac.uk)

meat are identified as major risk factors [7–9]. Despite the introduction of monitoring and control measures in most European countries as a consequence of the Council Directive 92/117 (Commission of the European Communities, 1992), *Salmonella* contamination in poultry in many European countries remains high [10–13]. In laying hens, reported infection rates in the European Union (EU) are decreasing, but large numbers of positive flocks are still detected. Southern Europe has the highest percentage of positive flocks, ranging from 5 to 10%. High percentages (between 3 and 8%) of positive table eggs are also reported in Southern Europe, while <1% of the eggs are positive in the rest of Europe. Egg products are less frequently positive in any country, as a result of processing. The most frequently reported serotypes in layer flocks in the EU in 2002 were Enteritidis (57·7%), Typhimurium (9·6%) and Infantis (6·9%). In table eggs, Enteritidis is even more predominant (72·9%) (Trends and Sources of zoonotic agents in the European Union and Norway, 2002). The level of contamination of poultry meat is very high in Europe. In 2002, approximately 10–15% of the poultry meat at the retail level was positive for many different serotypes in all European countries except Scandinavia (Trends and Sources of zoonotic agents in the European Union and Norway, 2002). As a consequence, at the end of 2003, the EU issued a regulation (Commission of the European Communities, No. 2160/2003) relating to *Salmonella* and other foodborne zoonoses obliging member states to monitor for *Salmonella*, reduce the risk of transmission of *Salmonella* and to put control measures in place in primary production. This regulation prohibits the sale of *Salmonella*-contaminated poultry meat and eggs after defined deadlines. Under these circumstances, there is an obvious urgent need for efficient control measures to be put in place in the poultry production chain. A variety of measures can be used to combat *Salmonella* in poultry. Eradication of *Salmonella* from poultry flocks and their environment does not seem to be a realistic option in most countries, due to the high contamination rate, the associated high cost of this action and the problems of environmental contamination. In addition to good management practice, hygiene on the farm and in the slaughterhouse is central to the reduction of entry of pathogens into the human food chain. Products that increase the resistance of the animal to infection or are antibacterial are, therefore, of increasing interest as components in any integrated control plans. Many of these products can help in controlling the infection but have been inadequately evaluated [14]. Vaccination is likely to take an increasingly central position in the control of *Salmonella* for the foreseeable future. Vaccination of breeder and layer flocks has been shown to confer protection against *Salmonella* infection and to decrease the level of on-farm contamination [15–30]. The application of an efficient vaccination strategy, however, requires a thorough knowledge and understanding of the epidemiology of *Salmonella* infections in poultry, together with an understanding of the efficacy and potential of the vaccines used.

Infection of chickens by *Salmonella* can occur by both vertical and horizontal transmission [31–34]. Although in all types of poultry production, infection by *Salmonella* can occur during any part of the production cycle [35, 36], it is likely that in both broilers and layers most of the initial infection takes place early post-hatch, as a result of hatchery contamination or persistent farm contamination. Infection of very young chicks results in high levels of environmental contamination and rapid transmission of pathogens as a result of litter contamination. This clearly illustrates the need for control products that confer resistance in the immediate post-hatch period whilst maintaining longer-term protective effects to last for the few weeks life of the broiler chicken and the longer period required for the layer. The broiler chicken has particular problems from the point of view of early protection. The generation of high-titre-specific maternal antibody lasts no more than a few weeks and, although there seems to be some protective effect against disease in the early post-hatch period, there is little effect on intestinal colonization by challenge strains [37–39]. Vaccination of young birds themselves has the disadvantage that the very young bird is immunologically immature [40, 41]. However, oral administration of *Salmonella* organisms to the newly hatched chicks not only induces an adaptive immune response, but is also able to confer, within 24 h of oral administration, a high degree of resistance against colonization and tissue invasion by other *Salmonella* challenge strains, through a combination of microbiological and innate immunological phenomena, which have potentially great practical significance. The aim of this review is to highlight and discuss these effects and review their potential for inclusion in vaccination control programmes.

IMMUNITY TO *SALMONELLA*

Immune responses to *Salmonella* depend on the host species and the *Salmonella* serotype infecting the host. Serotypes that usually induce a self-limiting gastroenteritis in a broad range of unrelated host species, while being capable of inducing systemic disease in a wide range of host animals, are called unrestricted or broad host-range serotypes. Host-restricted serotypes, such as *S*. Gallinarum in poultry, have a totally different pathogenesis. These bacteria cause a severe systemic infection which may result in the death of the animal. The pathogenesis of *S*. Gallinarum is characterized by its spread throughout the body and severe clinical disease with little intestinal involvement. Most data concerning immunity to *Salmonella* are derived from *S*. Typhimurium infection of mice, what is in fact a model of typhoid-like infections. It is, therefore, not always pertinent to extrapolate this information to non-host-specific *Salmonella* infections of poultry, such as *S*. Enteritidis infections. In the discussion below, data on immunity of mice are given since this is best understood, followed by available data on poultry immunity where this is known.

It is widely accepted that cell-mediated immunity is more important than humoral responses in protection against *Salmonella* [42, 43] even though most of these studies come from *S*. Typhimurium infection in mice where a typical typhoid-like infection is produced. How far this is true for disease-free gut colonization is unclear. In mice, Th1 cytokines, which enhance cell-mediated responses, are crucial for protective immunity against a primary *Salmonella* infection [44, 45]. Evidence for the importance of Th1 responses comes from experiments using IFN-γ receptor knockout mice and mice with neutralizing antibodies to IL-12, which are unable to resolve infection by an attenuated *Salmonella* strain, in contrast to mice lacking class-I-restricted T cells, $\gamma\delta$ T cells or Ig-producing B-cells, that are able to clear the infection [46–48]. Moreover, in mouse typhoid, protective roles have been shown for IL-1α, TNFα, IFN-γ, Il-12, IL-18 and IL-15, whereas IL-4 and IL-10 inhibit host defences against *Salmonella*, again pointing to the importance of the Th1 response in control of *Salmonella* [45]. In Ig$\mu^{-/-}$ knockout mice, lacking B lymphocytes, it has been shown that control of primary infection with avirulent *Salmonella* vaccine strains depends strictly on IFN-γ-producing CD4$^+$ T cells, whereas vaccine-induced protection against infection involves both cell-mediated and humoral responses, the latter in the later stages of infection [49–52]. Both cellular and humoral immune responses are stimulated by intraperitoneally administered heat-killed and live *Salmonella* vaccines in mice, the difference being the stimulation of Th1 or Th2 responses, which either direct B cells to switch to IgG2a via live organism stimulation of Th2/IL-4, or switching to IgG1 following stimulation of IFN-γ producing Th1 cells by killed *Salmonella* [53, 54]. IFN-γ has been found to be essential for reactive oxygen species-mediated killing of virulent *Salmonella*, although not essential for killing of avirulent vaccine strains [55].

In contrast to mice, in poultry little is known about immune responses to virulent and attenuated *Salmonella* strains. It is not possible to assess the role of Th1 in relation to Th2 immune responses, since there are, so far, no published studies involving Th2 cytokines. However, an important role of early CD8$^+$ T cells as a representative population of cell-mediated immunity was shown after primary *Salmonella* infection in young chicks [56]. It is proposed that cell-mediated immunity is more important than humoral responses for tissue clearance of virulent strains in poultry, while IgA responses and polymorphonuclear leukocytes seem to be the key players in intestinal clearance of *Salmonella* although this has not been proved experimentally and the evidence is confusing [57, 58]. Clearance of *S*. Typhimurium infection in chickens correlates with high cell-mediated responses (delayed type hypersensitivity reaction) and not with high antibody levels [59, 60]. In contrast, a study of Desmidt et al. [61] with *S*. Enteritidis-infected, bursectomized chickens showed that B-cell-depleted chickens have increased faecal excretion and higher caecal *Salmonella* counts, while having normal counts in internal organs, indicative of a protective effect of IgA against intestinal colonization. Colonization of liver and spleen decreased over time in control as well as in bursectomized animals, indicating that other immune mechanisms play a role in systemic clearance of *S*. Enteritidis in chickens [61]. The importance of cell-mediated immune mechanisms in systemic clearance of *S*. Enteritidis in chickens was recently investigated by Farnell et al. [62]. In this study, intraperitoneal administration of recombinant IFN-γ resulted in a decrease in organ colonization after oral *S*. Enteritidis infection.

Finally, in mice it has been shown that polymorphonuclear cells play an important role in resistance to *Salmonella* infections [63, 64]. In chickens

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heterophilic granulocytes accumulate in the propria mucosae of the caeca within 18 h following experimental infection with a *S*. Enteritidis field strain [65]. In infection with *S*. Typhimurium this is accompanied by acute enteropathogenic responses characterized by expression of CXC chemokines and a polymorphonuclear leukocytes heterophil (PMN) influx [66]. In response to *S*. Enteritidis, heterophils have been shown to up-regulate mRNA expression for pro-inflammatory chemokines IL-6 and IL-8 as well as the anti-inflammatory cytokine TGF-β4, whereas expression of IL-18 and IFN-γ was down-regulated [67]. The bacterial factors that are responsible for this effect have not been fully elucidated but in mice appear to include secreted effector proteins such as SopA, B and D [68], SipA [69] and the flagella protein FliC and probably FljB [70–72]. However, differences occur between different serovars, since the avian typhoid serovar *S*. Gallinarum down-regulates induction of IL-1 and IL-6 in avian epithelial cells [73]. There has been considerable discussion about the contribution to enteropathogenicity of PMN influx but there is now evidence that this does not contribute directly [74].

Granulocytopoenic (heterophil-depleted) chickens are much more susceptible to *S*. Enteritidis organ invasion, with the increase in bacterial number in the internal organs being proportionally related to the decrease in number of circulating PMNs [75, 76]. Another result underlining the importance of chicken heterophils in protection against *Salmonella* organ invasion was the finding that intraperitoneal administration of *S*. Enteritidis-immune lymphokines (SE-ILK) to 18-week-old chickens protected the animals from organ invasion by *S*. Enteritidis [64, 77]. SE-ILK are soluble products produced by T lymphocytes, derived from *S*. Enteritidis-immune hens, cultured in the presence of concanavalin A. Intraperitoneal administration of SE-ILK in chickens resulted in a dramatic increase in the number of heterophilic granulocytes into the peritoneum without changing the numbers of other leukocytes, and administration *in ovo* protected young chicks against organ invasion by *Salmonella* [78, 79]. Heterophil-depleted chickens showed a severe morbidity and mortality when a normally sublethal dose of *S*. Enteritidis was inoculated orally, further stressing the importance of heterophilic granulocytes [75]. These studies indicate not only the importance of this aspect of the innate response to *Salmonella* infection but also suggest that the course of infections might be modulated by manipulation of these responses.

VACCINES AND ADAPTIVE IMMUNITY

Vaccination against host-specific *Salmonella* serotypes, causing severe systemic disease in a particular host species (*S*. Gallinarum in poultry), induces a strong serotype-specific protective immunity against infection and disease [58, 80]. In contrast, vaccination against non-host-specific *Salmonella* serotypes has yielded variable success rates. The two infection types display very different epidemiological and pathogenicity patterns which, together with the nature of the immune response to systemic and intestinal infections, may account for these differences. Host-specific serotypes cause systemic disease with involvement of the monocyte-macrophage series and generally little initial intestinal colonization whereas the reverse is true for most of the serovars that are associated with entry into the human food chain causing food-poisoning [81, 82].

The efficacy of vaccine preparations is judged by the level of intestinal and systemic colonization and morbidity and mortality rates after vaccination and experimental infection using the oral or parenteral routes of administration are examined. However, the level of protection depends on the challenge strain, the route of administration, the infection dose, age of birds and species/line of birds. Consequently it is difficult to compare strictly the efficacy of the vaccine preparations currently available.

Vaccination against host non-specific *Salmonella* serotypes has had varying success. As a result of public interest this has been a fruitful area for research over many years. A number of reviews have appeared which summarize our knowledge and understanding up to 3–4 years ago [58, 83–85]. This is not the place to present a similar summary but rather to examine recent literature and to review current opinions on the use of different types of vaccine in poultry. This will be relevant to their use in other food animals and for any consequences for their application in young animals to exploit the early effects covered by this review.

Killed vaccines have been used to control host non-specific *Salmonella* infections in poultry with varying success. These have been used extensively as autologous vaccines and little information is available on their efficacy. Recent work [25, 86] supports earlier observations that they may be used to reduce mortality, although this is of little practical significance in the field. The relevance of this decrease in mortality for colonization of organs and shedding is also not

clear since *Salmonella* infection in the field is mostly asymptomatic. Different experiments with killed vaccines report variable effects on faecal shedding and colonization of the intestine and internal organs. Some work [87, 88] supports earlier contentions that maternal vaccination with bacterins does not significantly reduce excretion of *Salmonella* in the progeny although mortality can be reduced. However, positive results have been reported. Single oral or intramuscular immunization with formalin-inactivated *S.* Enteritidis bacteria, encapsulated in biodegradable microspheres, at 2 weeks of age, decreased faecal shedding and organ colonization of *S.* Enteritidis, after oral infection with 10^9 c.f.u. at 6 weeks of age [86]. Intravaginal vaccination with an oil-emulsion bacterin of *S.* Enteritidis at 38 weeks, followed by a booster 4 weeks later, reduced colonization of the ovary and spleen and reduced faecal shedding of a *S.* Enteritidis challenge strain [89]. After challenge, 36 out of 189 eggs (19·0%) in the vaccinated hens were positive, and this contamination rate was significantly lower than that in the unvaccinated hens (61/165 eggs, 37·0%). By contrast, in a field trial in which autogenous bacterins were used for single or double immunization, 10 layer flocks were vaccinated at different time intervals while one flock was left unvaccinated. The percentage of positive environmental samples and samples of internal organs of the vaccinated animals were not decreased relative to the animals of the unvaccinated flock [90].

A vaccine containing inactivated *S.* Enteritidis that was grown under iron-restricted conditions is available on the market in some European countries [91]. Also a vaccine containing *S.* Enteritidis as well as *S.* Typhimurium, both grown under conditions of iron restriction, is also commercially available [28]. Iron restriction is known to up-regulate bacterial factors that stimulate virulence and thus may stimulate important immunogens. However, given that the relevant genes are not up-regulated in macrophages [92] it might be more appropriate to produce the vaccines under the conditions experienced in that environment. The inactivated *S.* Enteritidis vaccine was efficient at decreasing egg contamination after intravenous challenge with *S.* Enteritidis [91]. This work is difficult to evaluate and oral challenge might have been more relevant. However, the combined *S.* Enteritidis and Typhimurium vaccine, when given intramuscularly at day 1 and week 4, decreased shedding after oral challenge with *S.* Typhimurium in a seeder-bird challenge model [28]. Fewer than 30% of the vaccinated birds shed *Salmonella* bacteria, while at 10 days post-challenge, more than 80% of the unvaccinated animals shed *Salmonella*.

Subunit vaccines have also been used in poultry. Outer membrane protein vaccines with adjuvant have been used to decrease shedding of *S.* Enteritidis in poultry [93]. Khan et al. [94] immunized 9-week-old chickens with two outer membrane proteins subcutaneously, followed by two booster immunizations with time intervals of 2 weeks. These outer membrane proteins were shown to be involved in attachment of *S.* Enteritidis to intestinal epithelial cell lines [95]. Immunization of either of the outer membrane proteins decreased caecal colonization $\sim$1000-fold when the animals were infected orally with 8×10^8 c.f.u. of a virulent *S.* Enteritidis strain [94].

Attention has been paid to the development of avirulent vaccine strains of *Salmonella* because of the accumulation of evidence that such strains of *Salmonella* are more immunogenic in mice and in poultry than are killed or subunit vaccines [42, 85]. Live vaccines have been tested extensively in mice and also in poultry. Although a number of different live *Salmonella* strains have been tested for their efficacy in experimental or semi-field studies only a few are registered and commercially available for use in poultry in Europe. The commercially available live *S.* Typhimurium and *S.* Enteritidis vaccine strains are either auxotrophic double-marker mutants derived through chemical mutagenesis [24, 96] or developed on the basis of the principle of metabolic drift mutations [17, 23, 97]. These are negative mutations in essential enzymes and metabolic regulatory centres as a consequence of which the resulting metabolic processes lead to prolonged generation times and corresponding reductions in virulence [97]. Some of these *Salmonella* live vaccines have been further characterized by molecular methods [98].

Another live vaccine registered for prophylactic use against *S.* Enteritidis (which was developed initially for immunization against *S.* Gallinarum) is the rough strain *S.* Gallinarum 9R [80, 99]. This vaccine strain has been tested more extensively in recent years since it has been shown to give cross-protection against *S.* Enteritidis, a member of the same serogroup. The extent of cross-protection against other serotypes, from either the same or other serogroups remains unclear. In a large field trial in The Netherlands in which 80 commercial flocks were vaccinated with the *S.* Gallinarum 9R vaccine strain, the flock level occurrence of *S.* Enteritidis infections was 2·5%

(2/80 flocks). This was significantly less than the flock level occurrence of *S*. Enteritidis infections in unvaccinated flocks (214 out of 1854 flocks; 11·5%) [27]. In 4500 eggs derived from five *S*. Gallinarum 9R vaccinated flocks, no vaccine strain bacteria were detected, while no evidence was found in another study for the faecal spread of the vaccine strain [26, 100].

Temperature-sensitive spontaneous *S*. Enteritidis mutants, able to grow well at 28 °C but not at 37 °C, have been tested as vaccine strains in poultry [101, 102]. When the mutant was orally inoculated (10^9 c.f.u.) in chickens at days 1, 2, 3 and 7 post-hatch and these animals were orally challenged at 7 or 14 days after the last vaccination with 10^8 c.f.u. of strains of *S*. Enteritidis and *S*. Typhimurium, fewer challenge bacteria were recovered from the caecal contents, liver and spleen 14 days post-challenge [101]. An alternative vaccination scheme (10^9 c.f.u. at day 1, and 2 weeks post-hatch orally) also decreased shedding and colonization of internal organs when the animals were challenged with 10^9 c.f.u. of a virulent *S*. Enteritidis strain 14 days after the last oral immunization [102]. As with many studies, challenge occurred soon after vaccination and the vaccine strain was still present in the tissues of 54 and 28% of the animals at the time of vaccination. Experiments such as these may be partially explained by the non-specific effects covered later by this review and all work involving short periods between vaccination and challenge must take into account stimulation of innate responses [103].

Numerous other live attenuated *Salmonella* vaccine strains have been developed by mutating genes involved in survival in host tissues. Genetic modification of the vaccine strain aims at reducing the risk of spread or persistence in the environment while at the same time inducing an adaptive immune response. It will be apparent (see below) that some of the mutations chosen may have consequences for the colonization-inhibition effect inducible in the gut of young animals. The complete genome of *S*. Typhimurium has been sequenced (www.genome.wustl.edu/projects/bacterial/styphimurium) and that for *S*. Enteritidis is now also complete (www.sanger.ac.uk/projects/*Salmonella*). This will facilitate the construction of completely rational mutations. Genes coding for metabolic functions or virulence factors are the main targets for producing safe vaccine strains. There is a certain rationale for inactivation of housekeeping genes which will reduce bacterial growth and virulence without greatly affecting the expression of key virulence determinants, required for appropriate immunogenicity [104]. Double or even triple mutations can be introduced to increase safety by reducing the risk of reversion by acquisition of genes by horizontal transfer [105, 106]. Whichever mutations are made, it would seem crucial that the vaccine strains retain the capacity of invasiveness in order to stimulate sufficient immunity to be protective. At the same time the vaccine strain needs to be eliminated before slaughter age in broilers, and before onset of lay in layer and breeder chickens. A number of genes have been mutated for the construction of candidate vaccines, including those involved in the biosynthesis of bacterial lipopolysaccharide (*galE*), regulation of expression of outer membrane proteins (*ompR*), amino acid or purine biosynthesis (e.g. *aro*, *pur*, *guaB*), regulation of carbon source utilisation (*cya crp*), virulence factors and many others, such as *htrA*, *phoPQ*, *recA* and *waaN* [107]. Few mutants have been tested in poultry [85], but the relevance of murine studies to poultry is questionable. For example, $phoP^c$ mutants of *S*. Typhimurium, although poor presenters of antigens *in vitro* [108], are highly immunogenic in mice [109, 110], this is largely ascribed to their persistent infection of and efficient presentation by dendritic cells, as opposed to their poor survival in macrophages [111]. How well such strains survive in chicken cells is totally unknown. *AroA* mutants have been tested extensively in poultry and found to be effective, albeit less protective than the 'gold standard' produced in chickens infected with a wild-type strain [112, 113]. Given the general consensus that there is little cross-protection between serovars, it is not surprising that Parker et al. [114] found no significant differences in egg or reproductive tract infection when laying hens were vaccinated at day of hatch, and 4 and 22 weeks with an *aroA* mutant of *S*. Typhimurium and challenged with *S*. Enteritidis 8 weeks after the final immunization.

Many of the characteristics and claims attributed to the *cya crp* mutant of *S*. Typhimurium, including the high level cross-protection, require confirmation and the mutant retains considerable virulence in gnotobiotic pigs [115]. Dueger et al. [116] also made claims for cross-protection using *dam* mutants, although the degree of protection was fairly small. These studies also highlight the shortcomings of mutations which demonstrate attenuation in systemic infection but are not tested for their ability to induce gastroenteritis. The exploration of the *sop* and other genes associated with Sip-dependent effector proteins [117] are a logical next stage in the creation of a truly rational vaccine.

The use of live attenuated *Salmonella* strains to deliver recombinant antigens to the immune system is an attractive additional strategy for the creation of multivalent vaccines for poultry. Multivalent vaccines would decrease the number of vaccinations in the field. Sustained expression of the heterologous antigen in the tissues in an immunogenic form at levels sufficient for priming a protective immune response is the main target when developing *Salmonella* recombinant vaccines [107]. Vaccination of chickens with a Δ*cya crp* mutant of *S*. Typhimurium expressing the *E. coli* O78 LPS O-antigens induced antibodies against the O78 LPS O-antigen and against *Salmonella*, and engendered a degree of protection against challenge with a pathogenic *E. coli* O78 strain [118]. Typhimurium vaccine strains have already been used as an antigen delivery system for oral immunization of chickens against two antigens of the coccidian parasite *Eimeria tenella* [119]. However, the delivery of antigens to the immune system is not sufficient *per se* to engender a protective response. A successful vaccination also requires the elicitation of an appropriate type of immune response. Thus, different groups are working on the development of carrier-based vaccination strategy in order to promote the optimal immune response. For example, strains carrying mutations affecting the specific course of infection can be exploited to modify the immune response elicited [120, 121] or the subcellular location of recombinant antigen in *Salmonella* vaccine strain may influence the type of the immune response [122]. In addition, the co-delivery of immune stimulatory molecules facilitates triggering a predictable response according to specific needs [123]. This type of work has, up to now, been performed only in mice. For example, Igwe et al. constructed a chimeric protein based on the *Yersinia* outer protein E (YopE) comprising the listerial antigens eliciting a cell-mediated immune response [124]. In mice orally immunized with attenuated *Salmonella* vaccine strains expressing the chimeric YopE translocated by the type III secretion system, this novel vaccination strategy led to the induction of a pronounced cytotoxic CD8 T-cell response that conferred some protective immunity [125].

A significant development in the last few years involves the use of *Salmonella* vaccines for the delivery of DNA vaccines. Such vaccines may induce immunity against the *Salmonella* carrier, heterologous antigen(s) from a second *Salmonella* serotype or other pathogen [126]. Consideration is being given to future modulation of the immune response by the co-expression of cytokines. A number of cytokines have been expressed in *Salmonella* vaccine strains, some of which have been shown to have an immunomodulatory effect, at least in mice [123, 127].

LIVE *vs*. KILLED VACCINES

As stated above, most data on vaccine-induced protection are derived from mice studies and care should be taken in extrapolating these data to poultry. Killed vaccines can be efficacious in reducing *Salmonella* in poultry. Nevertheless, live vaccines are considered to have advantages over killed vaccines. They stimulate both cell-mediated and humoral immune arms and expression of all appropriate antigens *in vivo*, while the latter stimulate mainly antibody production and express only the antigens present at the time of *in vitro* harvesting [42, 58]. Killed vaccines may also be destroyed rapidly and eliminated from the host, they may be poorly immunogenic in unprimed hosts and unable to induce cytotoxic T cells [57, 99]. Live vaccines have been shown to be more effective in increasing lymphocyte proliferation in response to *S*. Enteritidis antigens in laying hens [128]. They also have additional protective effects, particularly when administered orally, which can be exploited during their development and application. These include (1) genus-specific colonization-inhibition (competitive exclusion) demonstrated to be primarily an effect of microbial metabolism and (2) the stimulation of primed PMNs in the gut (see below). Killed vaccines are unable to induce these effects. It seems unlikely at the moment that more-effective killed or subunit vaccines will be produced in the next few years because many basic questions relating to identification of the major protective immunogens and the nature of the immune response in the chicken remain unanswered. Live vaccines have some disadvantages, including, perhaps most significantly, those associated with public acceptability. This is a major issue which should be addressed since the safety requirements are different for live vaccines than for inactivated vaccines.

The criteria for an ideal vaccine have been discussed previously [84, 129] and they include (1) effective protection against both mucosal and systemic infection, (2) attenuation for animals and humans, (3) efficacy in reducing intestinal colonization, and thus, reduced environmental contamination, and egg infection, (4) compatibility with other control measures and (5) cost-effective application. As indicated above, it is already possible to attenuate strains in a number

of ways but the inability to induce gastroenteritis is not always evaluated. It should be possible in the next few years to produce live, attenuated strains which are immunogenic for poultry and other food animals but which maintain attenuation in humans and other non-target species. This will, by necessity, require molecular genetics as a tool. The alternative is that live, attenuated vaccines are produced, as currently, by undefined chemical mutagenesis with strains possessing a combination of uncharacterized lesions whose cumulative effects only are known. The vaccines currently in use in Europe and elsewhere are very safe. It is an anomaly, however, that it is acceptable to allow their widespread dissemination while being seemingly over-cautious over the use of defined deletion mutants produced by genetic manipulation, even though each deletion is known and characterized. The environmental issues associated with the genetic modification of plants and also some food animals which may escape to the wild, are very different issues to the use of deletion mutants, with no additional DNA added. One advantage of the current widespread application of these vaccines is that data will now accumulate on any reversion and other potential risks to humans, target animals and the environment.

COLONIZATION-INHIBITION

Vaccination is regarded as an essentially prophylactic measure whose protective effect begins after a period of maturation of the B- and T-cell response. Thus, after vaccination of 1-day-old chicks, production of significant amounts of specific antibody responses against *Salmonella* takes more than 10 days [130]. For infections which may occur before this time, such as those arising from hatchery infection, this window of susceptibility is too long. However, orally administered live *Salmonella* organisms can induce a very rapid form of protection early in the life of the bird as a result of their colonization-inhibiting activity.

Colonization-inhibition, or competitive exclusion (CE), as it is more commonly known, can also be induced by the administration of normal gut flora preparations to newly hatched chicks. Young birds are highly susceptible to infection with *Salmonella*, because of the absence of a protective gut flora and immaturity of the immune system [41, 131]. The first can be overcome by the application of CE products based on cultures of normal flora obtained from pathogen-free adult birds [132], which, according to the recommendation of the WHO, should be applied as early as possible to 1-day-old chicks in the hatchery or by spraying eggs, rather than via the first drinking water. However, treatment with undefined flora is not permitted in many countries due to the potential risk of transmission of pathogens, although this can be avoided by appropriate testing of the product. The use of undefined flora and probiotics to control *Salmonella* in poultry will not be covered in depth in this review. For more information on this topic, the reader is referred to more specialized review articles [14, 133].

Because of some of the concerns associated with the use of undefined CE products, studies were initiated in the 1980s to search for bacterial strains which possessed the colonization characteristics of *Salmonella* but not their virulence attributes. Strains were sought in 109 environmental samples and amongst more than 600 individual strains of Enterobacteriaceae. A pool of three unusual strains of *E. coli* were isolated which when administered simultaneously, were partially effective at excluding *S.* Typhimurium [134]. During this study one group of 1-day-old chicks was found to be completely refractory to infection with the challenge of *S.* Typhimurium strain. This was because the birds had become infected with a strain of *S.* Montevideo from the feed soon after hatching. This strain, isolated from the birds and administered to a new batch of newly hatched chicks completely protected them against *S.* Typhimurium challenge 24 h later. In fact, it was found that an attenuated rough mutant of the *S.* Typhimurium strain also prevented establishment and colonization by the fully virulent, smooth parent strain [135]. This effect was, therefore, studied further.

Initial studies revealed that the effect required live bacteria; killed preparations administered either orally or parenterally had no effect. The inhibition was, therefore, not the result of a novel rapid immune response stimulated by bacterial antigens in the gut. Neither was it the result of bacteriophage activity. It was specific to related bacterial taxa. Thus, strains of *E. coli, Citrobacter, Proteus* and other related bacteria had no effect against *Salmonella* but did inhibit colonization by organisms from their own genera. Amongst the *Salmonellae*, not all strains were equally inhibitory. The mechanism was studied using an *in vitro* system of stationary-phase broth cultures [135, 136]. However, the practical aspects of the effect were immediately apparent and warranted further investigation. This [136, 137] showed that the protective

effect required high numbers of bacteria in the intestine and that as the normal flora began to develop the genus-specific exclusion reduced in efficacy. The effects were long lasting in terms of reduced faecal excretion and occurred in different chicken breeds, ducks [138] and with different diets. The effect became apparent after 6 h or so but only became fully effective after 18–24 h. Some strains were more effective than others, although no strain was fully effective against all *Salmonella* strains [137, 139], and there appeared to be a serovar-specific effect but how far this was related to clonality, rather than serovar specificity, remains unclear. The most profound level of inhibition *in vivo* occurred between isogenic strains. The fact that the challenge strains did not colonize also led to reduced invasion by them [140] and in the associated mortality (Barrow and Lovell, unpublished results). These data suggested that it might be possible to administer live vaccine strains to newly hatched chicks such that they would colonize the gut extensively and rapidly before the normal flora became established, and that this should induce a profound resistance to colonization by strains which may be present in the poultry house or may also have arisen in the hatchery. A search was made for a strain of *Salmonella* with a wide spectrum of inhibition, capable of preventing colonization by an extensive selection of strains. A strain of *S*. Infantis [141] and a strain of *S*. Hadar [140] were found to be more inhibitory than other serovars. These serovars are characteristically poorly invasive but highly colonizing [130, 142] and it may be that this latter characteristic is related to the inhibitory activity, possibly through a wide variety of nutrients available (see mechanism of inhibition below).

Attenuated live *S*. Typhimurium and *S*. Enteritidis vaccines with certain metabolic pathway mutations [16, 17, 23, 24, 27, 96, 143, 144] or deletions in genes for *cya* and *crp* [145] are immunogenic. However, it was also shown that these attenuated live *Salmonella* vaccines were generally not, or only briefly, able to inhibit intestinal colonization of homologous or heterologous *Salmonella* challenge organisms [65, 144, 146]. Thus, none of the currently available commercial live *Salmonella* vaccines is able to induce protection against *Salmonella* organisms by this exclusion or inhibition effect. There is, therefore, a need to identify live *Salmonella* strains which are sufficiently attenuated without affecting genes essential for colonization-inhibition. Recent studies confirmed not only the high level of attenuation of *Salmonella* strains with deletions in *phoP* but more importantly, demonstrated their colonization-inhibition ability [106].

Similar colonization-inhibition effects were also observed in the intestines of gnotobiotic pigs [147] suggesting that this is a general phenomenon not restricted to chickens. The occurrence of competition between related bacteria and its use in infection prevention has, in fact, been known for many years, although in most cases there is no understanding of its basis. It has been demonstrated between strains of *E. coli* in gnotobiotic mice and newborn infants [148] and between enterotoxigenic *E. coli* in pigs [148, 149]. This approach has also been used to reduce colonization of the skin by staphylococci [150–152], α-haemolytic streptococci [153] and also of the gut by *Clostridium difficile* in a hamster model [154]. Inhibition between skin staphylococci is thought to involve the production of antibiotic-like substances although there is no understanding of the mechanism of inhibition between the other bacterial types. Similar exclusion studies have been demonstrated between strains of *C. jejuni* [155] and work to determine whether the mechanism is similar in *Salmonella* and *Campylobacter* is underway.

The mechanism of colonization-inhibition is also poorly understood, and although an early hypothesis arose from the observation that a similar inhibition could be demonstrated in stationary-phase nutrient broth cultures, interactions with the host, either by competition for sites of adhesion or through stimulation of the innate immune system, have by no means been discounted. Of these mechanistic explanations neither explains completely the colonization-inhibition phenomenon, and both may be involved simultaneously.

BACTERIOLOGICAL EXCLUSION

The colonization-inhibition process was modelled *in vitro* by inoculation of small numbers of *Salmonella* strains in 24-h-old stationary phase nutrient broth cultures of another *Salmonella* strain or related bacteria [135, 156]. Continued incubation at 37 °C results in suppressed multiplication of the 'challenge' strain. *Salmonella* inoculated into broth cultures of strains from different genera were able to grow and vice versa. Even between bacterial strains of the same genus the greatest degree of inhibition was observed to occur between isogenic but antibiotic-resistant mutants. This *in vitro* system has considerable, although not complete, predictive value for inhibition

in vivo. [156]. Thus, serovars such as *S*. Hadar and *S*. Infantis showed a relatively wide spectrum of inhibition *in vitro* and *in vivo* [140, 141]. Studies conducted to elucidate the mechanism of bacteriological competition between *Salmonella* strains have been inconclusive. An initial hypothesis that the inhibition both *in vitro* and *in vivo* was the result of quorum sensing to suppress growth prior to starvation was not supported by mutational studies [157, 158]. This idea was stimulated by experiments in which the two strains were separated *in vitro* by a dialysis membrane such that physical separation was possible whilst still allowing diffusion of small molecules. Inhibition of growth of small numbers of bacteria inside a dialysis sac was prevented suggesting that either physical contact was required or that a signalling molecule normally generated in stationary-phase adhered to the membrane, as might occur if it was a peptide [136, 156]. However, initial analysis of the effects of random mutagenesis revealed that the specific *in vitro* inhibition was abolished by insertions in *nuoG* and *cydA* [159], genes encoding components of NADH dehydrogenase I and cytochrome d oxidase, both respiratory systems in use under reduced oxygen tension. This suggested depletion of nutrients or electron acceptors as a possible mechanism [159], although, interestingly, these mutants were fully inhibitory *in vivo*, suggesting oxygen is not an important electron acceptor in the chicken intestine. One of the genes found to be involved both *in vitro* and *in vivo*, was *atpB* and *atpH*, components of ATP synthase required under a variety of redox conditions. Analysis of a second transposon bank also showed involvement of respiration by the abolition of inhibition by insertions in *arcA*, *fnr*, *tatA*, and also that amino acid biosynthesis (*aroA*, *aroD*) and nutrient uptake and its regulation under low oxygen tension (*tdcC*, *sgaT*, *crp*, *dcuA*, *dcuB*, *aspA*, *speF-kdpE*) were involved. This suggested that bacterial growth to stationary phase *in vitro* or in the batch-conditions in the caeca results in nutrient depletion such that metabolically closely related bacterial 'challenge' strains are unable to grow whereas less closely related bacteria, using different carbon sources might be able to multiply [157, 159, 160]. Some insertions in flagella genes are also explainable in nutritional terms, facilitating movement towards higher nutrient and oxygen concentrations and it would be interesting to analyse the appropriate chemotaxis genes for this reason. Some mutations were less easily explainable by this hypothesis, such as *yhjH*, which showed sequence similarity both to diguanylate cyclase and to genes encoding signal transduction proteins, some of which may be involved in cell cycle regulation [157]. Most, but by no means all, of these growth non-suppressive mutants showed a similar phenotype *in vivo*, perhaps suggesting that under the different nutritional conditions in the gut, some genes, for example those required for respiration using oxygen as terminal electron acceptor, were not in use. Cell wall synthesis was also thought to be essential through the non-inhibitory activity of a *dapF* mutant, although lysis *in vivo* may have accounted for this phenotype [160]. The hypothesis of nutrient depletion as a mechanism of colonization-inhibition was not compatible with inhibition being produced by a broth culture but not by a filtered supernatant [135]. However, during filtration oxygen is added and if this is done totally anaerobically the growth of the strains in the filtered supernatant is very much less, unless additional nitrate is supplied (Turner and Barrow, unpublished results). An association with quorum sensing might have been strengthened by a link with genes known to be associated with this phenomenon, but mutations in *luxS* [161, 162] and *sdiA* [163, 164], known to be involved in this process in *E. coli* had no effect on inhibition [157].

The *in vitro* system involving inhibition by stationary-phase broth nutrient cultures of a freshly added strain is microbiologically very interesting and has practical value in explaining, at least in part, the *in vivo* phenomenon [157, 158, 160]. Despite the fact that the nature of the inhibitory action between two strains is not yet fully elucidated, it is clear that such interactions between *Salmonella* strains do play a role in colonization-inhibition *in vivo*.

One of the practical issues is that attenuation can introduce mutations which themselves abolish this effect and care must be taken to ensure this is maintained during attenuation [38].

COMPETITION FOR PHYSICAL SITES ON THE INTESTINAL EPITHELIUM

Attachment of intestinal pathogens to mucosal surfaces, thought to be the first step of infection, is mediated by bacterial adhesions which recognize specific receptors. Association with and invasion of the intestinal epithelium of the intestine has been demonstrated for *S*. Typhimurium [165]. *S*. Enteritidis has also been demonstrated to associate with the intestinal epithelial surface following oral inoculation of chickens. Such tropism may

involved several types of fimbriae or pili, and the *S.* Typhimurium genome encodes up to 12 putative fimbrial operons whose role has not yet been clearly defined.

The importance of adhesion in colonization control has been demonstrated with probiotic bacteria. Adhesion may be inhibited by blocking the receptor with specific adhesin analogues or by steric hindrance. *Lactobacillus* strains, which adhere to intestinal cells, have been shown to inhibit in a concentration-dependent manner, the adhesion to or invasion of either Caco-2 or HT-29 cells by *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, *Enterococcus faecalis*, *E. coli* and *S.* Typhimurium [166–168]. This competition has been demonstrated with viable lactobacilli but also with heat-killed strains and with their cell wall fragments [169]. In accordance with results observed *in vivo*, incubating Caco-2 cells with *Lactobacillus* strains was more effective before and during infection with enterovirulent *E. coli* than after infection [170].

Competition for binding sites within the gut is, thus, a possible significant component in colonization-inhibition. This hypothesis is supported by *in vivo* evidence. First, it is clear that the administrated protective flora colonizes the mucosa and can be seen as a mat of cells with the mucus or the glycocalyx. This could be an effective physical barrier to virulent *Salmonella* colonization [171, 172]. Second, although several [19, 139] studies indicate that full protection requires 24 h for complete efficacy, protection of chicks begins to become apparent within a few hours of administration of the protective strain [173]. Whether this is suggestive of a physical process of inhibition (binding inhibition) rather than any involvement of microbial metabolism or immune response remains to be seen.

Competition for receptor sites is unlikely to be the only factor involved in the protective process. The precise role of adhesion in the protective effect may never be completely determined because of the complexity of the gut as a habitat and the variety of interactions between host and microorganisms and between microorganisms themselves. Resistance to enteric pathogens may not solely be the result of the sum of microbial, epithelial, and immune factor effects, but is more likely to be the result of cross-talk between these factors. There are, for example, reports showing that several probiotic bacteria are able to inhibit the adhesion of pathogenic bacteria to enterocytes through their ability to increase the production of intestinal mucins [174].

HOST RESPONSE IN COLONIZATION-INHIBITION: A ROLE FOR GRANULOCYTES?

From these experimental studies there has been considerable argument as to how far the inhibitory effect was primarily a microbiological process or competition between related bacteria not involving a host response *per se*. However, other more recent studies have suggested that the host might be involved and have opened up further an area of infection-immune biology, which also has considerable practical consequences.

Since colonization-inhibition is a process that rapidly induces resistance to infection, adaptive immune responses are thought not to play a significant role. It is known, however, that immune cells are attracted very rapidly to the infection site after infection of chickens with virulent and attenuated *Salmonella* strains [65, 175]. After oral immunization of newly hatched chickens with an attenuated *S.* Enteritidis *aroA*, immune cells are attracted to the caecal lamina propria in high numbers [65]. These cells, comprising heterophilic granulocytes, macrophages, T lymphocytes and to a lesser extent B lymphocytes, infiltrate the caecal wall within 24 h post-vaccination, when up to 25% of the caecal wall may be occupied by these cells at this time. It was considered that these cells might conceivably play a role in colonization-inhibition, since the caeca are known to be the predominant site for colonization and invasion by *Salmonella* in the chicken [176, 177]. When birds were orally vaccinated with 10^8 c.f.u. of the candidate vaccine strain *S.* Enteritidis *aroA* CVL30 immediately post-hatch and subsequently challenged with the virulent homologous *S.* Enteritidis strain 1 day later, colonization of liver and spleen was strongly reduced during the first 5 days post-infection. However, on day 10 after infection there were no differences in the number of challenge organisms in liver and spleen between vaccinated and non-vaccinated animals. The caecal colonization by the challenge strain was only moderately suppressed in vaccinated birds compared to untreated controls [65]. This suggested that this cellular infiltration was not likely to be the main cause of colonization-inhibition, although this was not conclusively proven, but it did, however, demonstrate an interesting potential protective effect against

virulent *Salmonella* invasion soon after hatching. The same experiment was repeated in animals that were depleted of heterophilic granulocytes by the well-established model of 5-fluorouracil depletion [76, 178]. In this experiment the protection against colonization of internal organs was completely lost, suggesting a central role for heterophilic granulocytes in protection against invasion and organ colonization [178]. This is consistent with previous studies assessing the role of heterophilic granulocytes in protection against organ colonization by *Salmonella*. In this work, the extent of heterophilic granulocytic depletion was proportionately related to increases in the number of *Salmonella* in internal organs, and increasing the number of circulating heterophilic granulocytes following administration of cytokines derived from stimulated T cells protected against organ colonization by *Salmonella* [74, 76, 77]. Much older work had also shown that live vaccines can stimulate, within hours of inoculation, a high degree of protective immunity against homologous and heterologous bacterial challenge [80, 103, 179, 180], presumably through activation/priming of the innate immune system, once thought to be primarily macrophages [181], but perhaps more likely to be PMNs.

These data strongly suggest a role for heterophilic granulocytes in protection against internal organ colonization by *Salmonella* in chickens and also suggest that this is inducible by oral inoculation with live, attenuated *Salmonella* vaccines. This has considerable practical potential for poultry. The bacterial factors that are responsible for this effect have not been fully elucidated (see above). Similar results have also been found in mammals. A strain of *S*. Infantis was found to have a wide spectrum of colonization-inhibition against different *Salmonella* strains in newly hatched chicks [141]. This strain was also tested in gnotobiotic pigs to determine whether it would be similarly inhibitory against other serovars in young milk-fed mammals. This was found not to be the case. Although the *S*. Infantis strain was completely avirulent for 1-week-old pigs, it did not show colonization-inhibition against a fully virulent *S*. Typhimurium strain. However, the pigs pre-inoculated with *S*. Infantis and challenged with *S*. Typhimurium remained perfectly healthy [182], whereas pigs inoculated with *S*. Typhimurium only developed severe enteritis requiring humane killing. Similar results were found with a second *S*. Typhimurium challenge strain and *S*. Choleraesuis and when the experiments were carried out in gnotobiotic calves [74]. Of the cell types studied, only polymorphonuclear cells were observed in high number in the villi of the gut in the vaccinated groups. A more detailed study of this effect [74] concluded that the *S*. Infantis strains were sufficiently invasive to induce infiltration of large numbers of primed neutrophil granulocytes into the intestinal mucosa, which themselves did not induce any pathological changes, but which were highly antibacterial to the virulent *S*. Typhimurium strain inoculated 1 day later. In this context, pre-inoculation with attenuated *Salmonella*e may act similarly to commercially available Biostim [183]. Biostim is a glycoprotein derived from *Klebsiella pneumoniae* which has been shown to reduce the duration and rate of bacterial infection in the airways. The drug stimulates increased C3b and C3bi receptor expression in neutrophils [184], increases neutrophil phagocytic capacity [185] and increases neutrophil oxidative metabolism [186].

These three mechanisms appear superficially to be separate and distinct phenomena, two microbiological and the other involving the innate immune system, but both with practical implications for the use of live vaccines in young animals, including poultry. As indicated above it may be that these effects may operate simultaneously. However, the obvious differences may conceal a common thread which merits further exploration, namely that during colonization of the chicken caeca by *Salmonella*, these micro-organisms come into close contact with the mucosa, particularly in the region of the caecal tonsil. An assumption was made in early studies that intestinal colonization was primarily a reflection of bacterial metabolism, of whether or not the bacteria involved were able to exploit the nutritional and other physiological conditions present in the gut [187]. There is increasing evidence that this is not the case and that an interaction between colonizing bacteria and host is required as a component of colonization, whether or not this leads to extensive invasion and systemic disease. Colonization-inhibition may require all three mechanisms for full inhibition. The microbiological studies suggest establishment in the gut through appropriate metabolism and a failure to do this would prevent any interaction with the host, which may then take the form of a competition for adhesion sites or, where invasion takes place, involving heterophil activity, which may occur in or close to the lumen in the caecal tonsil. Thus, studies on these effects may also ultimately tell us a great deal about the mechanism of colonization and the extent to which host–pathogen interactions may be involved

in this aspect of infection which is central to food-poisoning.

CONCLUDING REMARKS

The ability of live *Salmonella* vaccine strains to induce colonization-inhibition and the neutrophil/heterophil induction effects are important and novel features that should be added to the list of desirable characteristics for the ideal vaccine for foodborne bacterial zoonotic pathogens. This will be especially important for the early protection of broilers but may have additional applications elsewhere.

As indicated above, a list of criteria can be produced with the properties of an ideal vaccine and, with current technology, it is possible to fulfil most of these criteria. First-generation live vaccines involving undefined and incompletely characterized mutants have been used for many years and are currently the only registered and accepted vaccines. Second-generation vaccines, in which genes known to attenuate *Salmonella* for systemic disease only (so-called rational vaccines) but which may have additional attenuating properties for humans, have been produced but their development has currently been halted by concerns over genetic manipulation. It should now be possible to produce third-generation live vaccines, which are truly rational, in which genes that have been identified as essential to systemic or gastrointestinal virulence can be deleted, while maintaining expression of key attributes required for invasion and immunogenicity, together with the additional beneficial characteristics described here.

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