***Salmonella* vaccination in pigs: a review**

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Abstract

The control of *Salmonella enterica* in pig production is necessary for both public and animal health. The persistent and frequently asymptomatic nature of porcine *Salmonella* infection and the organism's abilities to colonise other animal species and to survive in the environment mean that effective control generally requires multiple measures. Vaccination is one such measure, and the present review considers its role and its future, drawing on studies in pigs from the 1950s to the present day. Once established in the body as an intracellular infectious agent, *Salmonella* can evade humoral immunity, which goes some way to explaining the often disappointing performance of inactivated *Salmonella* vaccines. More recent approaches, using mucosal presentation of antigens, live vaccines and adjuvants to enhance cell-mediated immunity, have met with more success. Vaccination strategies that involve stimulating both passive immunity from the dam plus active immunity in offspring appear to be most efficacious, although either approach alone can yield significant control of *Salmonella*. Problems that remain include relatively poor control of *Salmonella* serovars that are dissimilar to the vaccine antigen mix, and difficulties in measuring and predicting the performance of candidate vaccines in ways that are highly relevant to their likely use in commercial production.

The challenge for *Salmonella* vaccination in pig production

Salmonella infection of commercially-produced pigs has been an ongoing issue for the industry for many years (Josland, 1954; McKinley et al., 1980; O'Brien, 1966), with public health protection becoming an increasingly dominant concern (Ojha and Kostrzynska, 2007). Source attribution studies for the European Union implicate poultry and pig meat as the commonest sources, after eggs, for human salmonellosis (EFSA, 2008). *Salmonella* serovars Typhimurium and Derby are commonly isolated from pig production worldwide, whereas the host-adapted *Salmonella* Choleraesuis is now rarely reported in Europe and Australia but is still frequently found in North America and Asia (Boyen et al., 2008; Gray et al., 1995). National prevalence values for *Salmonella* of between zero and 29% of pigs were reported from a recent systematic survey of ileo-caecal lymph nodes at slaughter in the European Union (EFSA, 2008). Other surveys of production herds in Asia, North America and Africa indicate, similarly, that carriage of *Salmonella* is common and typically not associated with clinical disease (Amaechi and Ezeronye, 2006; Eblen et al., 2005; Kim et al., 2010; Kishima et al., 2008; Rajic et al., 2005). Shifting patterns of *Salmonella* types have included the recent emergence on pig farms of monophasic DT193 and U302 strains closely related to *Salmonella* Typhimurium (EFSA Panel on Biological Hazards (BIOHAZ), 2010).

Whilst many pig production facilities practise high standards of biosecurity and segregation, a particular feature of *Salmonella* within the modern, integrated industry is its persistent presence within the breeding pyramid (EFSA, 2009; Letellier et al., 1999; Wales et al., 2013). This ensures that production facilities are continually at risk of importing *Salmonella* with new and replacement breeding stock. Whilst it is possible to achieve a 'clean break' at weaning from *Salmonella* in the breeding herd, this is difficult to achieve consistently in practice as new waves of different strains pass through the herd, undermining the 'herd-immunity' effect that limits transmission of *Salmonella* from lactating sows to young sucking piglets (Wales et al., 2011). Attempts to eliminate *Salmonella* from pig units also have to contend with the organism's resistance to removal from accommodation by conventional cleaning and disinfection methods (Funk et al., 2001; McLaren et al., 2011), and its frequent carriage on fomites and by wildlife (Barber et al., 2002; Berends et al., 1996). This means that the environmental persistence of *Salmonella* strains between batches of animals is very common, and may be the usual short-term determinant of the pattern of strains on a unit (Davies et al., 1998).

Salmonella vaccination may be used with the intention of controlling clinical disease, or to reduce subclinical shedding. Historically in Europe, and in the present-day in some parts of the world, it may be necessary to attempt control of outbreaks of clinical disease associated with a particular strain, commonly of the host-adapted *S. Choleraesuis*. In such a situation, the use of an autogenous killed vaccine prepared from the outbreak strain is a rapid intervention that may be effective, in concert with other control measures (Barrow and Methner, 2013; Roesler et al., 2006) if a licensed commercial vaccine is not available. By contrast, vaccination for *Salmonella* in modern pig production is usually employed to suppress shedding and to reduce infection pressure in the context of continuous herd infection, although a clinical enteritis problem amongst weaned pigs may in some cases provide the impetus to start vaccinating. Vaccination of commercial piglets to completely prevent *Salmonella* colonisation would require freedom from *Salmonella* amongst dams and in early age accommodation. This is currently not a realistic scenario, and in this respect pig vaccination differs from *Salmonella*

vaccination of poultry, particularly in egg production where elite and layer-parent breeding flocks typically are unvaccinated and intensively monitored for *Salmonella*. In this situation *Salmonella*-free layer and broiler parent poults are often protected by vaccination before possible exposure within accommodation (Davies and Wales, 2014).

Attempting to protect the consumers of pig products by controlling sub-clinical carriage and shedding of *Salmonella* using vaccination presents a substantially different challenge compared with vaccination for disease control. Firstly, there will be a variety of strains (and often serovars) which ideally should be controlled. These will often belong to differing antigenic groups and therefore issues of cross-protection may arise. Secondly, the desired effect will be a reduction of tissue colonisation and/or shedding by the time of slaughter, as opposed to protection from morbidity and mortality. This will likely involve stimulating effective immunity against broad-host-range serovars such as *S. Typhimurium*, which show a different pattern of invasion, multiplication and dissemination compared with host-adapted *S. Choleraesuis* (Boyen et al., 2008). Thirdly, any vaccine should not have any adverse effect on serological monitoring for *Salmonella* infection, where this is employed before or at the time of slaughter.

Measurement of the efficacy of any *Salmonella* vaccine in pigs needs to take account of the foregoing considerations about the intended use and required outcomes. For candidate vaccines to combat subclinical tissue invasion and shedding but without consequences for serological monitoring, it is necessary to decide on what constitutes a suitable study population, a natural versus an experimental challenge, and when and what to measure: morbidity, shedding, tissue invasion and/or load, plus serological responses (Denagamage et al., 2007).

***Salmonella* vaccination: general considerations**

Salmonella is a pathogen that invades the body through mucosal surfaces, and which is facultatively intracellular, exhibiting both intracellular and extracellular phases of its colonisation of tissues. Infection via the alimentary tract involves transit through enterocytes into the lamina propria of the gut, followed by entry into macrophages and subsequent dissemination to other tissues by a combination of cell migration and repeated cycles of cell death, release of *Salmonella* and re-uptake by phagocytes (Haesebrouck et al., 2004; Mittrücker and Kaufmann, 2000). Details of the degree and modes of tissue invasion appear to differ between host-adapted and broad-host-range serovars, and between host species (Boyen et al., 2008).

Humoral immunity to *Salmonella* infections has limited effect (O'Brien, 1966), because for much of the infection cycle the organism is within body cells, shielded from antibody action. Nonetheless (and unusually for cell-invasive bacteria) there is a strong humoral response to natural infection, including secretory IgA responses that may be effective in preventing initial invasion of the mucosa (McSorley and Jenkins, 2000; Mittrücker and Kaufmann, 2000). Cell-mediated immunity (CMI), characterised by a T-helper1 (Th1) lymphokine profile associated with activation of macrophages and cytotoxic lymphocytes, appears to be a critical part of effective anti-*Salmonella* immunity (Lindberg and Robertsson, 1983; Murtaugh, 2014).

Live *Salmonella* vaccines, presented to mucosal surfaces, theoretically offer the best combination of antigen presentation and co-stimulatory signals to elicit CMI and mucosal immunity (Haesebrouck et al., 2004). Indeed, such are its immunogenic properties and targeting to the mucosal immunoinductive Peyers patch sites that *Salmonella* has been genetically manipulated and used as an experimental vehicle for presenting viral, parasitic and other bacterial antigens, (Hur et al., 2012; Kim et al., 2012; Xu et al., 2012). Live attenuated *Salmonella* strains offer the additional advantage for pigs that inoculation can be performed by non-parenteral routes (principally in drinking water or by aerosol), without the need for handling and injection of individual animals.

However, effective mass vaccination with live vaccines can be technically demanding to accomplish reliably as there are many potential obstacles to achieving administration of a sufficient dose to all individuals. Live vaccines may readily be degraded or inactivated by inappropriate storage or reconstitution, or by interfering substances such as other micro-organisms or sanitisers in water supplies. Water lines of varying length, blocked or damaged drinkers, and individual animals' varied behaviours in respect of social hierarchy and drinking can all militate against a predictable distribution and uptake of vaccine in drinking water (Vermeulen et al., 2002). Such issues have led to attempts to incorporate vaccine with liquid feed rather than water (Edmonds et al., 2001).

There has also been a substantial effort over the years in developing killed *Salmonella* vaccines. This was a matter of utility and necessity in earlier decades when genetic attenuation techniques were in their infancy and *Salmonella* vaccination was largely a matter of controlling clinical disease associated with host-adapted serovars, particularly *Salmonella* Choleraesuis in pigs. More recently, killed vaccines have regained some appeal in view of their safety profile for consumers, predictable if labour-intensive dosing, and zero risk of reversion to virulence. This last issue is a potential hazard with live vaccine strains that, by mutation or acquisition of genetic material in the field, might reacquire virulent characteristics. Whilst experience with other licensed live vaccines, including *Salmonella* vaccines in poultry, has been reassuring in this respect, the development, testing and licensing of live vaccine strains is consequently more onerous than for non-living vaccines. Furthermore, advances in adjuvant technology and appreciation of the importance of growth conditions on suitable antigen expression has improved the effect, including on CMI, by killed vaccines (Barrow and Methner, 2013).

Technical aspects of candidate vaccine development

There are a number of strategies that may be used when trialling or implementing vaccination of pigs against *Salmonella*, and assessment of the vaccine protection needs ultimately to take account of its likely mode of use in the field. The age and production stages at which vaccination potentially can be used includes pre-weaning, weaner and grower stages for commercial rearing, pre-farrowing for generating passive immunity in piglets of farrow-to-finish herds, or regular vaccination of adults in breeding herds.

The dose, route and frequency of administration will depend on the nature of the candidate vaccine,

but is often the subject of a certain amount of trial and error. In theory, live vaccine strains should generate a strong immune response after one dose if they colonise and invade tissues to which they are applied. However, there is often a fine balance between sufficient attenuation and sufficient stimulation, and this may vary between serovars (Barrow and Methner, 2013; Coe and Wood, 1992; Kennedy et al., 1999; Linde et al., 1990). It cannot therefore be assumed that a single dose of a live strain will be sufficient. Killed vaccines are generally given by injection in two doses with adjuvant.

There are many approaches to the generation of candidate live vaccine strains by attenuation. Several defined-deletion mutants have been reported in the context of pig vaccination, as reviewed by Chu (2007) and Haesebrouck (2004). These include metabolic mutants with deletions affecting the *aroACD* aromatic amino acid synthesis or *purABEH* purine synthesis operons, the *galE* galactose-glucose metabolism gene, or the global regulator genes *cya* (adenylate cyclase) and *crp* (cAMP receptor protein). Deletion of the virulence plasmid *spv* genes following repeated passage through neutrophils has been reported (Kramer et al., 1992). Other mutations affecting survival in the body or invasiveness include *cpxR* (regulation of envelope stress response plus pili and fimbria) and *lon* (regulator of *Salmonella* pathogenicity island 1, affecting replication and survival within macrophages) (Hur et al., 2011; Hur and Lee, 2010), also *znuABC* zinc transporter operon (Pesciaroli et al., 2013). Other approaches to generating more or less well-defined attenuations include 'metabolic drift' mutations, with reduced growth rates linked to antibiotic resistance (Roesler et al., 2004), and auxotrophic strains generated by chemical mutagenesis that have specific growth requirements. The latter include the two 'Salmoporc' commercial vaccine strains that are unable to synthesize adenine. These also have additional attenuation, in the form of histidine auxotrophy or a stable rough phenotype for the 'STM' Typhimurium and 'SCS' Choleraesuis strains, respectively (Schöll and Grünert, 1980; Springer et al., 2001).

Under-attenuation can result in unacceptable clinical signs following administration, whilst over-attenuation leads to inadequate immune stimulation associated with poor *in vivo* survival, colonisation, or antigen expression by the vaccine strain. There are serovar differences in the effects of certain attenuations. As might be expected, genetic disruptions aimed at surface antigens can yield strains with greatly varying immunogenic effects (Leyman et al., 2011). Disrupting *cpxR*, which encodes a regulator of fimbria amongst other things, may increase antigen expression and immunogenicity (Hur and Lee, 2010). Attenuation may affect survival and growth of *Salmonella* in the environment too (Linde et al., 1990), which may be helpful in ensuring the absence of the vaccine strain by the time of slaughter.

Additional techniques trialled with live vaccines have included using *Escherichia coli* labile enterotoxin to act as an adjuvant for mucosal immunity (Hur et al., 2011), and using mixtures of live vaccine strains to address cross-protection against more than one serogroup. However, the latter approach has run into problems of interference between strains, causing reduced immunogenic effect (Barrow and Methner, 2013).

Attempts to use killed *Salmonella* vaccines to control clinical disease, either experimentally or in the face of natural infection, have met with mixed success (Arguello et al., 2013; Gradassi et al., 2013; Josland, 1954; Roesler et al., 2006). There are probably several factors that have influenced outcomes

in these studies, including variations in vaccination protocols (dose, frequency and route), preparations (growth conditions, inactivation method, adjuvant) and strains, both vaccine and challenge. A strong mucosal IgA response may be a significant element of vaccine protection against both colonisation and clinical disease (Roesler et al., 2004).

Traditional adjuvants use vehicles such as oil, emulsions or aluminium salts to immobilise antigens and enhance their presentation in tissues. Immuno-modulating adjuvants additionally, or alternatively, provide co-stimulatory signals to enhance the response in terms of type (Th1/CMI versus Th2/humoral) and intensity. In the long-established case of Freund's complete adjuvant this is achieved via the inclusion of killed mycobacterial material (Stills, 2005). Modern developments in respect of *Salmonella* vaccine adjuvants have attempted to enhance the Th1-type response, to lessen tissue reactions, and to provide adjuvant activity for orally-administered vaccines. Much work has yet to be translated from laboratory animals to livestock.

Co-stimulatory signalling of antigen-presenting cells occurs after activation of innate immunity through recognition of pathogen-associated molecular patterns by sensors such as Toll-like receptors (TLR). This can overcome mucosal tolerance of antigens on killed or subunit vaccines given orally (Rueckert and Guzman, 2012; Salman et al., 2009). The conserved domains of bacterial flagellin subunits (FliC and FljB) are ligands for TLR5 receptors, and are associated in mice with enhancement of an immune response to antigens presented as a fusion protein with flagellin (Huleatt et al., 2007; Qian et al., 2015; Yang et al., 2013), or as a separate molecule (Girard et al., 2011). The route of administration (oral, respiratory mucosal, injection) appears to affect the Th1/Th2 bias and mucosal (IgA) response obtained. *Salmonella* engineered to over-express flagellin showed increased immunogenicity in mice, as well as increased susceptibility to killing by phagocytes (Yang et al., 2012).

Other potential adjuvants for *Salmonella* vaccines include *E. coli* labile enterotoxin and Cholera toxin, which have immunomodulatory adjuvant effects in the gut probably associated with the promotion of antigen uptake and immune cell stimulation (Freytag and Clements, 2005). Efforts to circumvent the enterotoxic effects associated with labile enterotoxin have revealed adjuvant activity of the non-enterotoxic B subunit when engineered into a live *Salmonella* Gallinarum vaccine for poultry (Nandre and Lee, 2014), and also when administered trans-cutaneously (Fingerut et al., 2006; Tagliabue and Rappuoli, 2008). Although cholera toxin is well tolerated by pigs, compared with humans, its adjuvant effects on co-administered antigens appear to be modest compared with effects in mice, and rely substantially on close association between toxin and antigen (Foss and Murtaugh, 1999).

Polymer nanoparticles are another potential adjuvant, being taken up by antigen-presenting cells and showing an inherent capacity to stimulate some TLR classes (Tamayo et al., 2010). When antigen and co-stimulatory molecules (flagellin, mannosamine) were mounted on them, nanoparticles administered orally to mice promoted robust immune responses, shifted towards the Th1 pattern and mucosal IgA (Salman et al., 2009).

A specific issue with *Salmonella* vaccines for endemic, subclinical colonisation by broad-host-range

serovars is distinguishing immunological responses generated by vaccination from those due to ongoing cycles of infection. If vaccination is performed at an early age and is effective at reducing and preventing natural infection, this may not be a major issue as serological responses may be minimal by slaughter age, and indeed reduced compared with non-vaccinated groups if effective *Salmonella* control is achieved (Foss et al., 2013; Husa et al., 2009). However, if *Salmonella* is still circulating, vaccination can act as a ‘priming’ dose and result in a greater antibody response to the field infection. When it is necessary to show complete freedom from *Salmonella* infection, for example in breeding herds, then so-called DIVA (Differentiating Infected from Vaccinated Animals) vaccines are useful tools. These vaccines generally will lack specific antigens or epitopes (Leyman et al., 2011; Selke et al., 2007), and a serological test can be used to discriminate vaccine from natural infection.

Experimental studies

Vaccination of dams and/or pre-weaned piglets. There are a few studies, summarised by Wales et al. (2011), examining the protective effect on neonates and weaners of maternal vaccination aimed at providing passive immune protection against colonisation. Chu et al. (2007) reported increased and prolonged antibody in the serum of piglets born to dams given an experimental live attenuated *S. Choleraesuis* vaccine. A commercial live *S. Choleraesuis* rough mutant vaccine (“Suscovax”; Smith, 1965) was given parenterally to two sows at three and one weeks antepartum (Hanna et al., 1979a). It was associated in offspring with detectable somatic and flagellar antibodies and with partial protection against clinical signs and tissue colonisation by the same serovar, given as a challenge intranasal dose. A killed autologous vaccine of *S. Typhimurium* DT104 appeared to provide substantial protection when given in an intensive (oral plus parenteral) protocol to sows whose litters were subject to natural challenge (Roesler et al., 2006). Offspring of vaccinated sows had lower *S. Typhimurium* IgG and IgA titres than controls when examined at 16 to 20 weeks (suggesting less exposure to *Salmonella* after the waning of passive immunity) and zero isolations of *Salmonella* up to 142 days, with controls frequently yielding *S. Typhimurium* on culture. Results from a small study by Matiasovic et al. (2013) concur; a *S. Typhimurium* DT104 killed vaccine given in two doses antepartum was associated with reduced mucosal and tissue colonisation of piglets four days after dosing at three days of age with the vaccine strain.

Hur and Lee (2010) dosed pregnant sows orally with a defined attenuated *S. Typhimurium* strain (Δlon , $\Delta cpxR$) together with a derived strain producing labile enterotoxin. Offspring of vaccinated sows showed reduced clinical signs and *Salmonella* shedding when challenged with the virulent parent strain at one week of age. The two-dose trial administration protocols included parenteral killed and oral live doses, but only those where the second dose was oral live were associated with protection. Experiments using these same vaccines and sow protocols, plus vaccination of offspring at two weeks of age (parenteral, killed) and five weeks of age (live, oral) were reported by Hur et al. (2011). Humoral immunity was increased in piglets born to immunised sows, as assessed by IgG titres, but only piglets which themselves had been vaccinated were significantly protected from virulent challenge by *S. Typhimurium* at 11 weeks of age. Complete protection was only seen following vaccination of both sows and their piglets.

Eddicks et al. (2009) examined responses to oral administration of the 'Salmoporc STM' commercial double-auxotrophic *S. Typhimurium* strain at three and 21 days of age. By four weeks of age, seroconversion was noted and shedding of the vaccine strain had ceased, although it was found in tissues at up to six weeks of age. Further work on vaccination of both dams and young piglets with this vaccine was reported in a small study (nine sows, 58 piglets) by Roesler (2010). Oral vaccination of piglets at three days of age and at weaning (28 days) was followed by transient invasion of body tissues and shedding of the vaccine strain, without clinical signs. There was significant protection among vaccinates against clinical signs, shedding and tissue invasion following a challenge dose of *S. Typhimurium* DT104 at seven weeks of age. Interestingly, parenteral antepartum vaccination of the dams was associated with reduced tissue invasion of the vaccine strain in piglets, but this did not result in reduced protection against the post-weaning challenge dose. Indeed, excretion of the virulent DT104 strain was lowest among these piglets. Serological analysis suggested that neither vaccination of dams nor piglets would compromise serological monitoring at slaughter age. The authors suggested that, whilst active immunity was needed for protection of weaners and older pigs, humoral immunity in the gut and tissues provided by maternal vaccination should protect against early infection and reduce *Salmonella* challenge among young piglets.

Vaccination starting around the time of weaning. Several studies have examined vaccination of piglets within the one- to two-month age range. The immune cell population in the intestine of piglets resembles that of the adult by about seven weeks of age, although some subsets of cells (for example CD4⁺ lymphocytes) appear to be well-established sooner than this (Stokes et al., 2004; Vega-López et al., 1995).

Josland (1954) reported modest and variable clinical protection of weaners using formalin- or ultraviolet-inactivated *S. Choleraesuis* (three weekly doses) against heavy same-serovar challenge in small-scale trials. Lawson and Dow (1965) demonstrated protection afforded by an attenuated (rough) strain of *S. Choleraesuis* given parenterally at three to five weeks of age, against clinical signs and shedding following a challenge oral dose of the same serovar around 12 days later. Similarly, Smith (1965) reported marked reductions in clinical signs, mortality and enteric lesions among pigs injected with either of two rough mutant *S. Choleraesuis* strains at eight weeks of age, then challenged with the parent strain three weeks later.

Hanna et al. (1979b) examined the efficacy of a rough attenuated commercial *S. Choleraesuis* vaccine (Suscovax) derived from one of the above strains used by Smith (1965). There was reduced mortality and shortened phases of bacteraemia and nasal carriage following intranasal *S. Choleraesuis* challenge two weeks after Suscovax was given subcutaneously to seven-week-old weaners. Another undefined attenuated *S. Choleraesuis* strain was used by Kramer et al. (1987), given by injection or into the conjunctival sac of weaners followed by same-serovar challenge four weeks later. Significant reductions in clinical signs and systemic lesions were observed in vaccinated individuals over the following month, and there was a delayed cutaneous hypersensitivity to *S. Choleraesuis* outer membrane protein extract (consistent with a specific cell-mediated immune response) following vaccination.

A strain (SC-54) of *S. Choleraesuis* that had been attenuated by repeated passage through porcine neutrophils resulting in (amongst other things) loss of the virulence plasmid has been used in studies with young pigs. Roof and Doitchinoff (1995) administered it intra-nasally to four-week-old pigs and, following *S. Choleraesuis* challenge by the same route up to 20 weeks later, clinical signs were absent and tissue invasion was reduced in vaccinates compared with controls. Field studies by Kramer et al. (1992) confirmed the strain's efficacy against field challenge by *S. Choleraesuis* when administered into the nasopharynx of nursery pigs. This strain is at present commercially marketed in some territories, as 'Enterisol SC-54'.

Oral administration of a metabolic drift mutant of *S. Typhimurium* with mutations in *gyrA*, *cpxA* and *rpoB* to four-week-old weaners was followed by limited tissue invasion and a brief period of shedding but no clinical signs (Roesler et al., 2004). Vaccinated animals were relatively protected against clinical disease, shedding and tissue invasion following challenge with virulent *S. Typhimurium* DT104 three weeks later. Interestingly, vaccinates mounted a stronger IgA response to challenge than did unvaccinated animals, whose IgG responses were comparatively more intense.

In recent years, trials have often used vaccine candidates with defined attenuations. A double-attenuated (plasmid-cured, *crp* mutant) strain of *S. Choleraesuis* was given as a single oral dose to five-week-old weaners followed by a severe challenge with an unrelated field strain of *S. Choleraesuis* one to two weeks later (Chu et al., 2007). Mortality and severe morbidity was seen in challenged control animals. Milder clinical signs and lessened tissue lesions were seen among vaccinates, as was evidence of specific CMI in a lymphocyte proliferation assay. Kennedy et al. (1999) examined the effects of various attenuating *S. Choleraesuis* mutations upon protection against lesions and clinical signs following challenge. Against a background of $\Delta cya/\Delta crp$, loss of virulence plasmid or of muscle invasion capacity did not alter the vaccinal protection afforded, but attenuation of lipopolysaccharide synthesis did reduce protection.

An *aroA* mutant of *S. Typhimurium*, given as a two-dose injection starting at five to six weeks of age, was associated with reduced frequency of shedding of the (low virulence) challenge strain but similar mild clinical signs were seen in both the test and the unvaccinated control animals (Lumsden et al., 1991). In a related study using the same vaccine and challenge strains in similar-aged animals, post-challenge humoral responses (measured by agglutination assays) were similar between vaccinated and control animals, but vaccinates showed evidence of more CMI, measured by a *Salmonella* O-antigen-stimulated lymphocyte proliferation assay (Lumsden and Wilkie, 1992). Interestingly, despite litters being split and mixed, the source litter exerted a significant effect upon measured immune responses. Litter and sire effects upon CMI following $\Delta aroA$ *S. Typhimurium* vaccination were also seen in a follow-up study using the same assay (Lumsden et al., 1993).

Coe and Wood (1992) gave a single oral dose of a *cya/crp* mutant of *S. Typhimurium* to colostrum-deprived pigs around two months of age. Challenge with *S. Typhimurium* three weeks later was followed by serial post-mortem examinations. Animals only given the vaccine strain showed mild clinical signs (pyrexia and some soft stool) plus tissue invasion that was similar in extent and duration, but lower in intensity, than those given only the challenge strain. Animals vaccinated then challenged showed reduced severity of pyrexia and diarrhoea, plus a reduced duration of tissue invasion and

lower *Salmonella* counts in the ileum, than did controls. Therefore, efficacy as a vaccine was countered by limited attenuation in these highly-susceptible piglets. Similarly, Barrow et al. (2001) reported low attenuation of a *S. Typhimurium cya/crp* mutant (compared with an *aroA* mutant) for young colostrum-deprived piglets.

The commercial *S. Typhimurium* vaccine 'Salmoporc STM' has been the subject of several reports. Springer et al. (2001) administered the vaccine either in two oral doses or an oral plus an intramuscular dose, with oral challenge by *S. Typhimurium* DT104 five to six weeks later. Clinical signs were inconsistent in control animals but were reduced in intensity and duration in vaccinates. At *post-mortem* examination up to 10 days post-challenge *Salmonella* counts in the ileum and caecum were significantly reduced, although counts in intestinal lymph nodes were inconsistently reduced compared with control animals. Antibody titres in vaccinated animals around six weeks after the second dose were, almost without exception, below the cut-off point of a Danish meat-juice monitoring ELISA. Another study, using Salmoporc STM at four and seven weeks of age followed by *S. Typhimurium* challenge two weeks later, examined effects of a glucocorticoid dose (mimicking stress immunomodulation) given three weeks post-challenge. Post mortem examination one day after dexamethasone was administered showed a significantly reduced extent and intensity of tissue colonisation among vaccinated pigs. Salmoporc STM was also given as a two-dose course to weaners in a comparative trial using a strain with an antigenically-modified outer membrane protein (OmpD), as a candidate DIVA vaccine (Selke et al., 2007). A week after challenge with the same serovar, pigs given either the original or the modified vaccine strains exhibited protection against clinical signs of salmonellosis and had significantly reduced tissue *Salmonella* loads.

Two related studies have examined both transmission and susceptibility among vaccinated animals by using vaccinated seeder pigs to expose groups of young vaccinated pigs. Pigs were vaccinated orally at three and six weeks with Salmoporc STM (De Ridder et al., 2013a) or at four and seven weeks with Salmoporc STM modified by a deletion (*ArfaJ*) affecting lipopolysaccharide (LPS) synthesis (De Ridder et al., 2013b). Vaccinated seeder pigs were challenged two weeks later with *S. Typhimurium* and re-introduced into groups of vaccinates. Salmoporc vaccination was associated with significantly fewer pigs shedding the challenge strain than non-vaccinates, but the percentage of colonised tissues five weeks after challenge was not significantly different from untreated control animals. Use of the *ArfaJ* Salmoporc vaccine strain was associated with a significant reduction in the proportion of *Salmonella*-positive tissues six weeks after challenge, but only when it was used in conjunction with coated butyric acid in feed. This candidate DIVA vaccine strain additionally did permit discrimination between vaccinated and infected animals using a standard monitoring ELISA targeted at LPS antigens.

Several reports have discussed cross-protection following weaner-age pig vaccination. In a small study by Groninga (2000) a commercial live double-attenuated *S. Choleraesuis* vaccine was administered parenterally at three weeks of age, and pigs were challenged intra-nasally by a dissimilar serogroup (*Salmonella* Derby) two weeks later. Vaccination was associated with a significant (partial) reduction in tissue loads at *post mortem* examination between two and six weeks post-challenge. Charles et al. (2000) reported trials with a commercial *S. Choleraesuis* $\Delta cya \Delta crp$ vaccine (Argus SC) given to weaners in drinking water, who were subsequently challenged with *S. Typhimurium*. At a

higher challenge dose (10^{10} cfu/pig), clinical signs were ameliorated in vaccinated animals but shedding and tissue colonisation by the challenge strain was similar to controls. When the challenge dose was lower (10^6 cfu/pig), tissue colonisation was less frequent and extensive, and shedding was less prolonged and less intensive in vaccinated animals.

Cross-protection was also examined by Husa et al. (2009), who reported that two live attenuated *S. Choleraesuis* commercial vaccines (Enterisol SC-54 and Argus SC/ST, both previously discussed) administered orally to month-old pigs were associated with reduction of clinical signs following intra-nasal challenge with *S. Typhimurium* six weeks later. Qualitative isolations from tissues *post mortem* did not differ significantly from controls. One further study (Foss et al., 2013) used three-week-old pigs given attenuated (Δ *cya* Δ *crp*) mutants of either *S. Typhimurium* or *S. Choleraesuis* orally, and then challenged three weeks later with a non-attenuated *Salmonella*. The challenge strains, variously, were one of: the same serovar, the same serogroup, or a different serogroup from the vaccine strain. Reductions in challenge strain shedding and tissue colonisation at *post mortem* examination four weeks later were seen with both vaccines, but the degree of protection lessened as the antigenic similarity between vaccine and challenge strains diverged, despite measures of humoral and cell-mediated responses to challenge appearing similar.

Vaccination of older growing pigs. A *S. Typhimurium* bacterin given orally in two doses did not protect fattening pigs against clinical signs or tissue invasion by experimental same-serovar challenge, although shedding was reduced (Gradassi et al., 2013). A novel recent approach to attenuation has involved mutating the zinc transport operon (*znuABC*) in *S. Typhimurium*, to produce a non-persistent strain that provokes mild pyrexia only, when administered to three-month-old pigs (Pesciaroli et al., 2013). Such a strain should provoke, but not survive, an inflammatory response. Its one-dose administration to three- to five-month-old pigs was associated with significant reductions in clinical signs, shedding and tissue invasion following virulent *S. Typhimurium* challenge (Gradassi et al., 2013).

Field studies

Salmonella vaccination studies involving natural challenge in field situations are far less common than small-scale single-dose challenge studies with short-term endpoints. An early field trial (Josland, 1954) failed to demonstrate any clinical protection associated with inactivated *S. Choleraesuis* (10^9 cfu with alum adjuvant) given to weaners. The challenging serovar(s) were undefined and the vaccine was given as a single injection. Palyusik (1964) injected a formalin-inactivated *S. Typhisuis* vaccine to dams and weaned pigs (single dose) plus unweaned piglets (two doses) in a field trial of over 5000 animals in units where swine paratyphoid disease was established. Substantial but partial protection against clinical disease and mortality was observed.

Arguello et al. (2013) conducted a field trial using an inactivated *S. Typhimurium* DT104 vaccine (10^{10} cfu, adjuvanted with aluminium hydroxide) injected into grower pigs at around 10 and 13 weeks. For *S. Typhimurium*, there were significant reductions in shedding after vaccination and in colonisation of mesenteric lymph nodes and caecum at slaughter, although *Salmonella* of other

serogroups was not significantly reduced. A recent trial of an autologous monophasic *S. Typhimurium* vaccine, also inactivated and adjuvanted with aluminium hydroxide, used a subset of sows and piglets on a multisite farrow-to-finish unit, with mixing of vaccinated and unvaccinated pigs at the post-weaning stage (Ruggeri et al., 2015). Immunisation of sows ante-partum plus piglets (at four and eight weeks) provided the most consistent reductions in shedding and tissue contamination at slaughter. Some protection was seen where immunisation was performed at only one life stage, but results were more variable and lacked statistical significance.

Rough mutant *S. Choleraesuis* strains were given by injection to eight-week-old weaners on a farm with endemic *S. Choleraesuis* disease (Smith, 1965). Post-weaning mortality was more than halved in vaccinated versus control groups, whilst evidence of systemic *S. Choleraesuis* infection was seen in around four times as many control as vaccinated animals. A trial covering eight farms (12,000 pigs) with endemic *S. Choleraesuis* infection, using the Enterisol SC-54 attenuated *S. Choleraesuis* strain administered at around 10^9 cfu per individual in drinking water, was reported by Kramer et al. (1992). Clinical cases were not seen after vaccination was started, and a clinical outbreak on one unit was halted rapidly. The same SC-54 (C1 serogroup) vaccine strain was trialled in a field study dosing piglets once, on the first day after birth in a breeding/finishing integration with predominantly B-serogroup endemic strains (Schwarz et al., 2011). At slaughter, individual-level seroprevalence and mesenteric lymph node colonisation among test animals (45% and 33%, respectively) were modestly but significantly decreased compared with control animals (80% and 60%, respectively).

A further trial used another commercial *S. Choleraesuis* vaccine (Argus SC/ST; *Δcyd Δcrp*) given in drinking water at three and 16 weeks of age to pigs on a unit with diverse endemic *Salmonella* serovars (Maes et al., 2001). Compared with unvaccinated controls, there were significantly fewer (0.6% vs, 7.2%) *Salmonella*-positive ileo-caecal lymph nodes among animals at slaughter. However, seroprevalence near slaughter age was higher than, or similar to, control animals depending on whether the ELISA was interpreted with a higher or a lower sensitivity, respectively, using optical density cut-off values of >10% versus >40% of positive control.

Summary and conclusions

Salmonella vaccination in pigs has to contend with an antigenically-diverse pathogen that, once within tissues, can largely evade antibody-mediated attack. Evidence from experimental challenge studies indicates that CMI and secretory (IgA) humoral responses to vaccination are important in providing significant levels of protection against clinical and subclinical consequences of *Salmonella* exposure. However, strong mucosal immunity provided by passive transfer of colostral immunoglobulin may be sufficient alone to prevent *Salmonella* colonisation of pre-weaned piglets.

Most trial reports focus on vaccinating young pigs and use a single high-dose challenge followed by monitoring and sampling for days to a few weeks in order to assess vaccine effects. Many candidate vaccines have proven efficacious in terms of preventing or reducing clinical signs of salmonellosis associated with a serovar matched to the vaccine, and a large field trial involving units affected by *S. Choleraesuis* has borne this out. Reports of trial vaccines using serovars other than *Choleraesuis* or

Typhimurium are rare.

There is, however, comparatively little information on the abilities of candidate vaccines to reduce or eliminate *Salmonella* carriage and shedding by the time of slaughter, which is when *Salmonella* in pig production may potentially lead to *Salmonella* contamination in pig products, resulting in human disease. Findings from a study where challenge doses were varied suggests that vaccination that protects against clinical signs but not tissue invasion, carriage and shedding after a high-dose *Salmonella* challenge, may in fact reduce tissue invasion and shedding when the challenge dose is lower. This is supported by another study where *Salmonella* was isolated least frequently from animals where vaccination and feed interventions were combined. Nonetheless, there remains a substantial gap in the data regarding outcomes that are significant for public health protection. Given the sub-clinical endemic nature of much *Salmonella* infection of pigs in modern production systems, the principal driver for the use of *Salmonella* vaccination is likely to be perceived benefits in herd *Salmonella* status at marketing. Widespread adoption of vaccination for this purpose will require better evidence than currently exists.

Killed vaccines, with appropriate administration protocols and adjuvants, have shown protective effects against antigenically-similar strains in experimental challenge and field studies, and additional strategies (such as using enteric toxins or nanoparticles as adjuvants for orally-presented antigen) may extend the usefulness of this approach. Furthermore, some live vaccines have additionally been able to demonstrate a degree of cross-protection against differing *Salmonella* serogroups in both small-scale challenge and field trials. On current evidence, lipopolysaccharide O-antigen appears to be of high importance in stimulating effective protection, and the degree of protection seen lessens as the somatic antigens of challenge strains diverge from those of the vaccine strains. Where autologous bacterins are to be used, careful sampling followed by analysis of multiple isolates may allow the selection of a strain (or strain mix) that includes surface antigens present on many or all of the prevalent serovars, according to published antigenic formulae (Grimont and Weill, 2007). However, the partial nature of protection afforded by candidate and licensed vaccines in all studies is consistent with the view that the elimination of *Salmonella* from production herds by vaccination alone is probably over-optimistic.

Protection of very young piglets from *Salmonella* colonisation holds out the prospect of very substantial reductions, or complete elimination, of *Salmonella* load at the start of rearing. Despite the immaturity of the piglet immune system, present evidence indicates that a combined strategy of maternal vaccination followed by early vaccination of pre-weaned piglets is needed for best protection against challenge in the post-weaning period. Antibody titres from colostral passive immunity are minimal by around eight weeks of age (Wales et al., 2011). The identification of significant effects of litter and sire upon piglet vaccine protection (Lumsden et al., 1993; Lumsden and Wilkie, 1992) suggests that genetic and/or environmental factors may influence the outcome of vaccination programmes.

There are a number of areas where innovation may make distinct contributions to advances in *Salmonella* vaccine creation and delivery. Mucosal delivery routes, such as intranasal spray (Braucher et al., 2012; Feng et al., 2013), can enhance cell mediated and local immune responses, and need to

be optimised for delivery in modern pig production systems. There has been some success in producing efficacious experimental DIVA vaccines that may prove especially useful in high health status breeding herds, as relevant investigations have mostly indicated that the effects of non-DIVA vaccines upon current regimes of serological monitoring at slaughter may be neutral or beneficial. However, more stringent monitoring criteria associated, for example, with assurance schemes may make DIVA vaccines more attractive for production herds in the future. Other vaccine technologies that may prove useful, but which have not yet been reported in pig *Salmonella* studies, include ghost cell preparations (Szostak et al., 1996) and purified antigen subunit preparations. *Salmonella* is regarded as a promising vector for the delivery of DNA vaccines (Bartolomé et al., 2010; Ingolotti et al., 2010), which may therefore facilitate the development of vaccination against *Salmonella* itself via this technology.

The core region of surface lipopolysaccharide is particularly conserved in *S. enterica* (Heinrichs et al., 1998), more so than for *E. coli*. This provides a possible avenue to address the challenge of inducing effective cross-protection against diverse serovars, as some rough mutants (with core regions and other outer membrane antigens exposed to immune receptors) have proved to be immunogenic and partially protective against heterologous serovar challenge in mice (Nagy et al., 2008; Nnalue et al., 1999). Thus, strains with partial or conditional suppression of O-antigen might achieve both increased cross-reactivity and, if used as live vaccine, a suitable degree of attenuation. Another potential avenue for exploration is the use of rationally attenuated *Salmonella* that has been genetically engineered to express other antigens, as a vector organism for a multiplex live vaccine, as briefly discussed earlier. A potential problem with this is that exposure to field *Salmonella* infection may lead to an immune response that limits the uptake and response to subsequent vector vaccines. Nonetheless, this approach could address the problem of serovar cross-protection by involving diverse antigens from a range of *Salmonella* serovars, or indeed use antigens relating to other porcine pathogens.

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