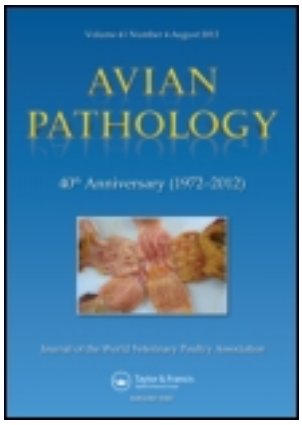

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The long view: Salmonella - the last forty years

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REVIEW

The long view: *Salmonella* – the last forty years

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As a part of the 40th anniversary celebrations of *Avian Pathology* we review the last four decades of *Salmonella* research which has led to major progress in our understanding of the bacteriology and infection biology of the organism through the huge advances in molecular biology and immunology that have accompanied technical advances in biology generally. In many countries combinations of improvements in management, sometimes under legislative pressure and supported by a number of basic biological interventions, have resulted in reductions in incidence in the *Salmonella* serovars that are commonly associated with food-poisoning to unprecedented low levels in parent flocks, broilers and layers. Utilisation of the information generated during the past few decades should improve the efficacy of surveillance and biological interventions both for the intestinal carriage that is associated most frequently with human infection and also for systemic diseases, including fowl typhoid and pullorum disease. These two diseases continue to be major economic problems in many countries where the possibilities for improvements in hygiene may be limited but which, nevertheless, are increasingly a significant part of the global economy in poultry meat.

Introduction

Salmonella and poultry have been linked epidemiologically and, more recently, economically since the 1930s (Buxton, 1957). This review for the 40th Anniversary of *Avian Pathology* is written by scientists who have been active in *Salmonella* research in different ways for more than 30 of the past 40 years and who have had direct links with Houghton Poultry Research Station, and particularly with H. Williams (Willie) Smith, whose research on *Salmonella* dated from the early 1950s and who developed the 9R vaccine for *Salmonella* Gallinarum in 1956, with J. F. Tucker and indirectly with Bob Gordon who did a huge amount to eliminate Pullorum disease from UK flocks in the 1940s–60s.

Our scientific understanding of the organism has exploded in the past 25 years (Neidhardt *et al*. 1996) with the increasing application of standard bacterial genetics, initially developed for use with *Escherichia coli,* and more recently with the expansion of whole genome sequencing which is continuing to generate fascinating information as you read this. Similarly, the more recent increase in immunological knowledge and tools in mammals, particularly in man and mouse, has now extended to the chicken, even though the chicken has always been crucial to our early understanding of the function of fundamental components of the immune system. The development of immunological tools for use with poultry and the availability of the chicken genome sequence have enabled us to begin to understand, in a completely rational way, how the bird responds to infection. In the vast majority of cases the research relates specifically to the chicken with still very little work having involved turkeys or ducks.

Despite this huge expansion in our knowledge of *Salmonella* as a pathogen, approaches to control during this 40 year period have been largely empirical and in some cases, such as the use of antibiotic therapy, have been ill advised. Having said that, the combination of rational approaches to reducing exposure through improved hygiene and management, coupled with improved monitoring procedures and, in some cases, vaccination, have been effective in Europe in reducing infection with the major serovars to levels not seen before.

The expansion of the poultry industry into South America and the Far East, not only to feed their growing and increasingly wealthy population but also for export, has resulted in a globalised economy with transmission of old and new pathogens between trading countries. In some of these countries high ambient temperature currently restricts the imposition of strict controls on the environment and hygiene. It is in these situations where biological approaches to control, whether by vaccination or by other more novel approaches, might find a more important role in reducing infection.

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Taxonomic status

Together with a greater understanding of the basis of infection and immunity there has been a constant shifting of sands in the taxonomy and nomenclature of the serovar/serotypes of *Salmonella*, which were once traditionally referred to as species. During the past 40 years this process has gone through several iterations with the current understanding developed by Leon Le Minor and Michel Popoff in 1987 of two species within the *Salmonella* genus, *enterica* and *bongori* based on DNA similarity. The *S. enterica* species is divided into several subspecies including *arizonae* and *diarizonae* (previously separate species) and *enterica*, which now includes all the serovars of most interest to veterinary and human medicine. As these are not given species status their names are not now italicised. Thus the old *Salmonella enteritidis* is now referred to in full as *Salmonella enterica* subsp. *enterica* ser. Enteritidis or abbreviated to *S.* Enteritidis. But, after all, what is in a name - the disease and economic and public health problems remain the same?

Salmonella on the world stage

Salmonella serotypes come and go, often introduced or becoming prevalent through changes in husbandry or other practices. Thus the appearance of *S.* Agona in the 1950s was associated with increasing use of South American fish meal as a feed constituent. Forty years ago saw the appearance of *S.* Hadar in turkey flocks and subsequent spread into broiler flocks. The biggest epidemiological change worldwide during the last 40 years has been the rise and fall of *Salmonella* Enteritidis. *S.* Enteritidis infection of eggs became one of the first major food safety scares in the 1980s. The initial association with large outbreaks implicated eggs as a major source of infection even though the highest levels of infection were found in broilers.

The peak of the *S.* Enteritidis epidemic in the UK was in the late 1980s to early 1990s and involved several phage types which spread worldwide. However, the source and reason for its appearance has resulted in considerable speculation. This included the suggestion by Wolfgang Rabsch and his colleagues in 2000 that the policies developed to control *S.* Gallinarum and *S.* Pullorum in the 1950s allowed *S.* Enteritidis to emerge in chicken flocks in the 1960s, prior to which immunity to the antigenically-related host-specific serovars had prevented its establishment. However, in the UK the predominant *S.* Enteritidis phage type 4 (PT4) emerged in the early 1980s many years after the demise of the avian host-specific serovars. Evolutionary changes in PT4 or its introduction through breeding flocks imported from mainland Europe may have been the cause of the epidemic in the UK. It should also be remembered that improvements in surveillance and diagnostic testing mean that it is not always possible to compare historical data with information obtained in more recent years.

As a consequence of the *S.* Enteritidis epidemic, which resulted in high national publicity and accompanying political fall-out, the UK and the Netherlands led the way towards EU-level legislation (Directives 92/117, 2160/2003 and 1168/2006) to require monitoring for *S.* Enteritidis and *S.* Typhimurium, initially of breeder and layer flocks and eventually also of broilers and turkeys. The intention was to improve feed quality and ultimately to introduce a requirement for national control measures involving surveillance, biosecurity and vaccination. Each EU member state was required to develop and implement a series of National Control Plans (NCP) for *Salmonella* and to set out targets for its reduction. These plans were produced at different speeds in different countries and the legislation appears to be having a positive impact in reducing *Salmonella* levels within the EU. *S.* Enteritidis declined from a peak of hundreds of infected flocks and over 20,000 human cases in England and Wales in 1992 to a handful of positive flocks and less than 5,000 human cases by 2010. When considering PT4, the main cause of egg-associated infection in the UK, the decline has been even more marked, with a drop from over 15,000 human cases in 1992 to 459 in 2010. The reduction of *Salmonella* in UK egg production is a clear success for control strategies such as the 'Lion Mark' scheme introduced in 1998 and which included improved surveillance, hygiene and biosecurity and perhaps, most significantly, vaccination of laying hens.

Despite the successes in control in the UK and other countries, *S.* Enteritidis remains the most important serovar associated with foodborne salmonellosis worldwide.

Regular baseline surveys carried out by the European Food Safety Authority (EFSA) have given a clear snapshot of *Salmonella* in European poultry with prevalence in egg production falling across Europe and varying between 1% in the UK and 14% in Spain. Figures for 2008 show that infection rates in broilers varied from $<1\%$ in Scandinavia (these countries were the first to adopt rigorous, even draconian, controls for *Salmonella* in poultry production in the 1970s) through 14.9% in Spain to 85% in Hungary. Serovars such as *S.* Infantis and *S.* Hadar are becoming more prevalent in a number of EU countries.

Unlike the EU, surveillance of *Salmonell*a in the USA is more fragmentary and it is more difficult to get a clear picture, although available evidence indicates that *Salmonella* infection remains a considerable problem. United States Department of Agriculture (USDA) figures suggest that as much as 23% of US poultry meat is still infected with *Salmonella* (www.fsis.usda.gov). No specific strategy is in place for control, which may reflect State rather than Federal decision making. The situation in Australia is more interesting since, although around 13% of carcasses are contaminated with *Salmonella* at slaughter, more than half of these are *S.* Sofia, a serovar with a low potential for virulence in humans.

Countries such as Thailand, which have rapidly expanding industries for both domestic consumption and export, have recognized the problem and are developing strategies for the surveillance and control of both *Salmonella* and *Campylobacter*, though little information is available on *Salmonella* prevalence. In many other countries *Salmonella* remains a problem of secondary animal or human health significance.

No doubt the increasing globalisation of trade in poultry meat will increase pressure on producing countries to introduce targets for reducing levels of infection which will comply with the expectations of the consuming countries. This will inevitably lead to reduced levels of infection in an increasing number of countries.

Turkeys remain a relatively minor source of infections for man even in countries such as the UK. The levels of infection in ducks are generally not monitored and in the Far East, where these are major livestock species, they must act as a major source of human infections. There is scope for a considerable amount of work to be carried out in this part of the world which is increasingly a source of poultry meat for other countries.

Extensive use of chemotherapeutic antibiotics for disease control and growth promotion, prior to implementation of the 1969 Swann report (Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine) in the UK and Europe, led to a fear of increased resistance in poultry *Salmonella*. However, this has not been a problem in poultry as it has in the calf rearing industry with resistant strains such as *S.* Typhimurium DT104, which nevertheless have infiltrated the poultry industry. Other serovars associated with poultry, notably *S.* Infantis and *S.* Virchow, are also frequently resistant to a range of antimicrobials and the increasing use of quinolones against *S.* Enteritidis has now led to increases in resistance. The uncontrolled use of antibiotics in many countries has resulted in multi-drug resistance in many serovars including *S.* Gallinarum and *S.* Pullorum. Greater control over the use of antibiotics in animal rearing within the EU should result in reduced levels of resistance, although this will take place only slowly. Unforeseen consequences such as increases of clostridial infection in broilers have resulted from EU-wide withdrawal of glycopeptide antibiotics used for growth stimulation. This is an additional problem with obvious implications for global trade involving countries where control of antibiotic use is not as restrictive as it is in the EU.

Fowl typhoid and Pullorum disease

Both Fowl typhoid (FT, caused by *S.* Gallinarum) and Pullorum disease (PD, caused by *S.* Pullorum) were largely controlled in developed poultry industries in Europe and North America through 'test-and-cull' policies employed in the mid 20th century. However, significant outbreaks leading to considerable losses have been reported both within the UK and mainland Europe in recent years, although it is unclear if the outbreaks were related to 'natural' infection or factors associated with the use of the *S.* Gallinarum 9R vaccine. Certainly the increasing appearance of extensive organic farming has increased contact between poultry and the environment with a return of many 'old' diseases including PD and FT.

The impact of FT and PD is harder to judge in developing industries due to a lack of systematic surveillance, but their importance is clear, from both published and anecdotal evidence, with FT outbreaks in Mexico, Argentina, Nigeria and India. Endemic FT and PD are still found in many countries in both commercial production and backyard flocks including countries with expanding industries such as Brazil and South Korea where there has been considerable research activity in recent years.

Control of FT through vaccination with the *S.* Gallinarum 9R live vaccine has been used in countries such as Korea. There have been reports in the UK of problems associated with its use after a largely untarnished reputation for 50 years. More rational approaches to attenuation are now being taken to generate live vaccines with defined sets of mutations rather than relying on the technologies used to generate BCG (Bacillus Calmette-Guérin vaccine for human tuberculosis) after the first World War.

Diagnosis

In a review of limited length this is perhaps not the place to discuss progress in diagnosis in detail. The OIE (World Organisation for Animal Health) continues to review current and new methods for identification and surveillance. Suffice it to say that evidence of the presence of the bacteria is still regarded as the gold standard. Methods based on the detection of bacterial DNA, such as PCR, are increasingly being introduced for identifying the presence of the bacteria in samples with the proviso that this does not necessarily indicate the presence of live bacteria. Similarly serology (ELISA) can be used for flock monitoring to indicate exposure of the flock to the organism. Technologies such as microarrays are also being explored to replace standard serotyping methods.

The microbial basis of infection and disease

Our increasing understanding of the molecular and microbial basis of *Salmonella* pathogenesis in poultry (largely chickens) has occurred in parallel with, or slightly in the wake of discoveries using the murine model of salmonellosis which has been used most frequently over the last six decades. It has gradually become accepted that, although this may be a good model of systemic diseases such as fowl typhoid in chickens, it does not accurately represent the biological events occurring in the gut or elsewhere in the chicken. It has been clear for both species that the outcome of infection is the combined effect of the microbial gene set and the host genetic background, which was shown for chickens in the 1950s.

One of the major features of salmonellosis is host-specificity, the basis of which continues to elude a scientific explanation. Much confusion has resulted from the fact that the various forms of disease/infection caused by the various *S. enterica* serotypes - systemic disease, gastro-enteritis and disease-free intestinal colonisation - have not been treated separately, so that like has not been compared with like. There is little evidence that intestinal colonisation is host-species restricted but some evidence that it occurs in gastro-enteritis.

Prior to the early 1970s phenotypic differences between serovars associated with different forms of disease were known but were not understood. These differences included the absence of type-1 pili from serovars that produce systemic disease, auxotrophy in these serovars and the absence of motility in the avian *S.* Gallinarum and *S.* Pullorum, but research workers lacked a means to tease apart the genetic composition of each serovar. Suitable techniques were developed for laboratory *E. coli* strains in the 1950s but these were of little value for field strains. Thus attempts by Willie Smith to transfer virulence characteristics from *Salmonella* strains by F plasmid-mediated Hfr transfer came to nothing. It was

only when the ability to tag plasmids with transposons was realised that the contribution of a large plasmid - found first by Garth Jones and colleagues in 1981 in *S.* Typhimurium and later by Matthew Binns and Paul Barrow and his group in *S.* Gallinarum, *S.* Pullorum, *S.* Enteritidis and other serovars - to systemic disease began to be understood. However, the so-called virulence plasmid did not contribute to colonisation ability, which remained a focus of research for avian salmonellosis, since this was a key attribute enabling food-poisoning serotypes to contaminate carcasses.

Intestinal colonisation

The use of more extensive transposon mutagenesis was possible from the late 1980s using Tn*5* and derivatives such as Tn*phoA* (allowing identification of insertion in genes encoding surface functions) which insert almost randomly around the chromosome such that libraries of mutants could be created, each with a separate insertion. The use of Tn*phoA* by Steven Craven in 1994 led to recognition for the role of LPS in colonisation of the chicken intestine and screening larger libraries of Tn5 mutants individually in chickens by Keith Turner and the Barrow group at the Institute for Animal Health (IAH), Compton, assisted in identifying a role for miscellaneous regulatory bacterial functions in colonisation. The requirement for large numbers of animals to screen these mutants led to the development of molecular sequence tagging of individual mutants (signature tagged mutagenesis, STM) such that up to 96 mutants could be inoculated simultaneously into an animal and each could be recognised individually in the gut or faeces. One STM study carried out by Duncan Maskell, Tim Wallis and the Cambridge and IAH groups, investigating colonisation in both the calf and chicken, indicated the contribution of Pef, Csg, Sth and Stb fimbria, LPS, Salmonella Pathogenicity Island (SPI)-3 and a variety of central metabolism and transport genes to colonisation in birds. Similar studies have been carried out more recently in Korea with *S.* Gallinarum. More comprehensive screens using techniques such as TraDIS (Transposon-directed insertion-site sequencing) could be used to ascertain the role of every gene through the generation of thousands of overlapping mutations.

Before the use of these techniques there was little knowledge of the mechanism of colonisation. It was known that the caeca were the main site of colonisation and, following the identification of microbial adhesion to the duodenal mucosa in porcine *E. coli* enteritis, adhesion was proposed as a mechanism of caecal colonisation. However, in an organ where the flow rate of chime was very low this seemed an unnecessary attribute. Nevertheless, the STM screen indicated that attachment to host mucosa may be involved via the fimbriae. This might perhaps involve the caecal tonsil where the host is able to control entry and exit into the caeca in both naïve and immune animals and where there is the possibility of a very intimate interaction between host and pathogen.

More recent comparisons of genome sequences obtained from colonising serovars such as *S.* Enteritidis with the closely related non-colonising *S.* Gallinarum, published by a consortium led by Nick Thomson at the Sanger Institute, have revealed additional features of interest including an accumulation of pseudogenes in operons associated with metabolism of carbon compounds. These compounds include 1,2-propanediol (*pdu*) and ethanolamine (*eut*), both breakdown products of host membranes from cells shed into the gut, and tetrathionate (*ttr*), the electron acceptor used for catabolism of propanediol and ethanolamine in the gut, using cobalamin as a co-factor (*cob* and *cbi* operons, which also contain pseudogenes in *S.* Gallinarum). Similar changes are found in *S.* Typhi when compared with *S.* Typhimurium and provide an indication of important metabolic pathways during colonisation of the gut. Genomic analysis of an increasing number of *Salmonella* strains with different biological characteristics has also shown the importance of horizontal transfer of genes between strains including virulence determinants such as the *sopE* gene, which is important in gastroenteritis.

Another recent alternative approach to identifying colonisation genes has arisen from a more complete knowledge of the genome, enabling the production of whole open reading frames or oligonucleotide microarrays to follow changes in gene expression during colonisation in comparison with bacteria harvested from broth cultures. Recent studies by Barrow, Mike Jones and colleagues of the colonisation of newly hatched chickens by *S.* Typhimurium and *S.* Enteritidis confirmed a role for 1,2-propanediol, ethanolamine and propionate as carbon sources and also SPI-1, SPI-2 and SPI-3 and fimbrial (*stf*, *stb*) genes, many of these indicating a close interaction with host cells. Most bacterial growth appeared to take place at the mucosal-lumen interface where nutrient and electron acceptor concentrations would be higher.

Systemic disease

Similar rates of progress have been made with systemic infections using STM in *S.* Gallinarum by Joon-Seok Chae and his group in South Korea identifying a number of recognised and new SPIs. Simple allele replacement studies by Mike Jones and Paul Wigley have shown that SPI-2, responsible for preventing the maturation of the phagolysosome in *Salmonella*-infected macrophages, was essential for systemic virulence in *S.* Gallinarum as it is for *S.* Typhimurium in mice but that SPI-1, responsible for the ability to invade non-professional phagocytic cells, was not. Survival and multiplication within macrophages is a key aspect of systemic virulence although the holistic picture is probably far more complex since *S.* Dublin organisms in calves at least translocate from the gut to the spleen as extra-cellular bacteria. In the later stages of disease the bacteria re-enter the intestine through areas of necrosis in the mucosa. This stage is poorly understood, as is the nature of the sterile areas of myonecrosis in the heart produced by *S.* Gallinarum and *S.* Pullorum in chronically infected birds. The two avian serovars Gallinarum and Pullorum are also characterised by persistent infection following convalescence from disease early in life. It is known that Pullorum persists within macrophages and the immune response to the organism is also interesting and different to that following infection with *S.* Typhimurium (see below). It is not known which bacterial genes facilitate this persistence or whether it is absence of functional genes that is responsible.

Other individual virulence characteristics that have been investigated include the huge array of fimbriae elaborated by most serovars, up to 13 in many, and how far they contribute to colonisation and virulence. Such investigations are complicated by the fact that inactivating individual fimbria by mutation does not necessarily produce large reductions in virulence and there is a train of thought that considerable redundancy may exist.

The contribution of flagella to virulence is also complicated by the absence of flagella in *S.* Gallinarum and *S.* Pullorum, although interestingly the flagellin gene *fliC* in these serovars are not pseudogenes. These two serovars were found by Pete Kaiser and the IAH group to induce a much weaker inflammatory response in the gut following infection when compared with *S.* Typhimurium and *S.* Enteritidis. The interactions between flagellin and innate immunity via TLR5 is an interesting new area of exploration studies by Adrian Smith and the IAH group and may have potential for vaccine/adjuvant development.

Gastroenteritis

The discovery in the 1990s that *Salmonella* produce secreted proteins in response to increases in temperature above ambient and that they are secreted through a Type Three Secretion System (TTSS) prompted interesting work on the role of these systems and their effector proteins in enteritis. This work was carried out very elegantly by Tim Wallis and Ed Galyov at IAH using the ligated intestinal loop model adapted from earlier work by Willie Smith in the 1960s on the pathogenesis of enterotoxigenic *E. coli*. This resulted in a greater understanding of the cellular events surrounding invasion and uptake of the bacteria by epithelial cells within minutes of contact with the mucosa. Others, including the group led by Andreas Bäumler, have expanded on this work by determining that the process involves a non-fimbrial adhesion, the translocation of up to 15 effector proteins via the TTSS into the host cells which induces actin rearrangement promoting bacterial entry. Once in the cells, proteins such as SopB, an inositol phosphate phosphatase, increase chloride and fluid secretion.

No doubt such studies will, in due course, be reassessed by full sequencing of total RNA from biological samples. Deep-sequencing certainly generates huge amounts of data and is a very powerful tool.

It seems obvious from the above very brief summary that, although so much has been done using the tools of bacterial genetics and more recently whole genome and deep sequencing, to provide answers to so many microbiological questions that appeared intractable 40 years ago, we still await the translation of the more recently generated information into more rational vaccines and other approaches to controlling these and related pathogens.

Immunity to *Salmonella*

During the past 40 years our understanding of immunity to infection with *Salmonella* has grown in parallel with developments in avian immunology aided by access to genomic resources and the ability to perform molecularly-defined studies of the avian immune system (for those interested in avian immunology we recommend the text Avian Immunology, edited by Davison, Kaspers and Schat, 2008). It has not been possible to progress studies on immunity to pathogens in chickens as fast as those achieved in mice resulting from the increasing availability in the last 30 years of knock-out and other transgenic mice. This has thus limited many studies in chickens to monitoring changes in individual immunological parameters during infection. However, many of the basic components in mice and chickens are similar, allowing valid comparisons and predictions to be made in terms of function of individual components, such as the impact of Th1/Th2 bias in the immune response. Furthermore, over the past few years germ-line transgenic birds developed by Helen Sang and others have become a reality and, although costs may preclude the generation of a resource as effective as the rodent systems, these technologies herald a new era in avian immunology. Some classical technologies, for example *in ovo* bursectomy, deserve more widespread use. The use of genetically resistant and susceptible lines of birds is an approach that has been employed by several European groups to correlate the outcome of infection with particular response profiles. Their use has also facilitated "genetic mapping" experiments which have been critical in defining parts of the avian genome (and sometimes identifying candidate genes) that are associated with early clearance of *Salmonella* (reviewed in Calenge *et al.*, 2010). Advances in high throughput molecular techniques (e.g. microarray and parallel sequencing technologies) further add to our capacity to define molecular responses and probe their association with specific genotypes. We are now confident that application of the new technologies, with careful experimental design anchored in the basic biology of *Salmonella* infections, will accelerate the discovery pipeline to define the mechanisms of immunity.

It has to be remembered that *Salmonella* can inhabit a variety of niches within poultry including the gut lumen as well as the deeper host tissues (in intracellular and extracellular locations). Extrapolation from studies on *S.* Typhimurium infection (typhoid) in mice in the 1970s by colleagues such as Charles Nauciel, Carlos Hormaeche and others led to the paradigm of the dominance of T cell-mediated immunity in clearance from the tissues. In chickens it should in theory have been possible to tease this apart relatively easily in the 1960s by using surgical bursectomy and thymectomy, although the latter can be technically difficult.

The mechanisms that operate against *Salmonella* infection in the gut lumen are quite different to those that operate in the tissues. Most serovars such as Typhimurium and Enteritidis in birds of more than 3 days old are either prevented from translocating across the gut epithelium or killed quickly at the site of entry as a result of the inflammatory response. In contrast, with serovars Pullorum and Gallinarum there is a much reduced inflammatory response in the gut and more substantial colonisation of sites outside the gut tissue.

Effective immunity requires the activation of macrophages and polymorphonuclear (PMN) cells (largely heterophils in birds). Epithelial cells, tissue resident macrophages and dendritic cells in the gut are activated by the recognition of conserved "pathogen associated molecular patterns" (PAMPs) using pattern recognition receptors (PRR) such as the Toll-like receptors (TLR) which have been identified in chickens. In *Salmonella* the

major PAMPs are lipopolysaccharide (LPS), flagellin and unmethylated CpG motifs in the DNA. TLR4 is activated by LPS, TLR5 by flagellin and although TLR9 (which recognises CpG motifs in mammals) is not present in the chicken genome this recognition capacity is fulfilled by chicken TLR21 (laboratories of Greibel and van Putten). All of these TLR-PAMP interactions are important for the induction of responses in a range of cell types including epithelial, macrophage and PMN cells. Interestingly, the finding by Adrian Smith and IAH colleagues that genetically modified aflagellate *S.* Typhimurium was better able to cross the gut, led support to the idea that TLR5-flagellin interactions are an important event in restricting flagellate serovars (Enteritidis and Typhimurium) largely to the intestine. This may also partly underpin the ability of non-flagellate serovars (Gallinarum and Pullorum) to rapidly escape the gut and colonise deep tissues. Indeed, these serovars stimulate different responses in a variety of cell types (Kaiser, Wigley, Jones and colleagues). The magnitude of heterophil and macrophage responsiveness to *Salmonella* or their extracts were found by Christina Swaggerty, Mike Kogut and colleagues to differ in genetically resistant and susceptible birds indicating their role in protection.

A range of cytokines and chemokines have been shown by groups in Cambridge and IAH (UK) to be transcriptionally up-regulated during different phases of infection with different *Salmonella* serovars and in different ages or lines of chicken. Pro-inflammatory cytokines such as IL(interkeukin)1β and IL6 are characteristically elevated during the early phases of infection and this is often followed by an up-regulation of mRNA encoding interferon-γ (IFN-γ). During the past few years Pete Kaiser and colleagues have determined the repertoire of chemokines in the chicken (which is somewhat different to the mammals) and understanding how these are involved in co-ordinating recruitment of cells to sites of infection will be important in coming years.

The adaptive immune system of all jawed vertebrates comprises B cells, T cell receptor (TCR)γδ + T cells and TCRαβ + T cells. The numbers of TCRγδ + T cells were found by Angela Berndt and Ulrich Methner to alter during infections with *Salmonella* but the function of these cells remains a mystery. In contrast, the biology of TCRαβ + T cell subsets are better understood in many vertebrate species and the chicken is no exception. TCRαβ + T cells expressing the CD4 co-receptor are restricted to peptide in the context of major histocompatibility (MHC) class II molecules and are also known as T-helper (Th) cells. A range of subsets of Th cells have been identified in mammals including the Th1 and Th2 subsets originally defined by Tim Mosmann and Robert Coffman in 1986. The response can be distinguished by the production of IFNγ by Th1 cells and IL4 alongside other cytokines including IL6 and IL13 for Th2 cells. These cytokines exist in chickens and responses can become biased towards Th1-type or Th2 type responses. TCRαβ + T cells expressing the CD8 co-receptor are restricted to peptides presented by MHC class I and are also known as T cytotoxic cells. Chickens contain both CD4 + and CD8 + TCRαβ + T cells and these appear to function similarly to those found in other species. In contrast, there are important differences in the MHC molecules and how this system functions in birds; Jim Kaufman has led much of the work in this area and has coined the term "Minimal Essential MHC" for the organisation of these molecules in birds.

The profile of infection with many serovars suggests a major role for adaptive immune responses in clearance of primary infection and in the enhanced clearance of secondary infection. Similarly, the effectiveness of live attenuated vaccines in protection against *S.* Gallinarum or *S.* Enteritidis also indicates a pivotal role for adaptive immunity. Strong T cell responses are stimulated by exposure of birds to infection with various *Salmonella* serovars which have been found to peak at the time of clearance. The T cell response is dominated by cells producing IFNγ, which is consistent with a Th1 type response, although CD8 + TCRαβ T cells as well as TCRγδ T cells and Natural Killer cells also produce IFNγ. The IFNγ-dominated type of response is very similar to responses found in mammalian hosts infected with *Salmonella*. B cell responses to infection are similarly strong with very high levels of *Salmonella*-specific antibodies (IgM, IgY and IgA) produced, peaking at around the time of clearance. The potential for antibodies to participate in clearance of serovars such as Typhimurium from the gut was considered likely since the gut lumen can only be affected by a subset of the immune mechanisms that operate in systemic sites. Adrian Smith and colleagues employed surgical bursectomy (SBx) to create B cell-deficient birds and tested this hypothesis. The SBx birds were unable to mount an antibody response against infection with *S.* Typhimurium but suffered no problems in controlling or eliminating this infection. The rate of clearance from the gut of surgically bursectomised birds paralleled that of intact chickens during both primary and secondary infections. These data offer an important lesson; a strong response does not mean an effective response (even when logic indicates that this is a likely outcome). In contrast, chickens that were treated with cyclophosphamide during the first few days post hatch (another widely used method of rendering chickens B cell deficient) then infected at 6 weeks of age were less able to clear *Salmonella* from the gut. This suggested that the cyclophosphamide-treated group experienced a deficiency in a non-B cell subset that could not recover post-treatment. Interestingly, exposure of the SBx or cyclophosphamide-treated birds to a second challenge led to the same rate of accelerated clearance seen with intact chickens. These data suggest that the mechanisms employed by chickens to control enteric *Salmonella* differ between primary and secondary infection and that neither is dependent upon B cells or antibody.

In some specific cases clearance from the tissues is not complete and *S.* Pullorum is able to persist in the tissues until sexual maturity. Wigley and colleagues at IAH found that the pathogen persisted in splenic macrophages in young convalescent birds until onset of lay when a transient immunosuppression associated with a surge in sex hormones in the female enables the bacteria to escape and infect the oviduct resulting in vertical transmission. This group also found that the immune response induced by *S.* Pullorum is associated with higher levels of IL-4 and reduced IFNγ indicating a Th2-type response in contrast to the more common Th1-type response associated with serovars such as *S.* Typhimurium.

Approaches to controlling *Salmonella*

The understanding of *Salmonella enterica* serovars as a major source of food-borne zoonotic infection has resulted in a parallel application of control measures already in use elsewhere in veterinary or human medicine or by developing totally new approaches, some of which have proved to be very successful.

Antibiotics. It is hardly surprising that chemotherapeutic antibiotics were used from the 1960s to reduce mortality in young birds, caused by a variety of pathogens including *Salmonella* and *E. coli*. Extensive use has always generated concern over the selection for resistant bacteria, in most cases mediated by transmissible plasmids. Experimental work from 1966-1974 by Willie Smith, John Tucker and John Walton showed that transmission from *E. coli* to *S.* Typhimurium could occur in the chicken gut and that the drugs also disrupted the gut flora, which enhanced *Salmonella* colonisation with the result that birds became more susceptible to re-infection. Even the more recent use of quinolone antibiotics generates resistance which is chromosomal and mutational, but which nevertheless can be transmitted by bacteriophage. Resistance has never been the problem in poultry that it was in calf rearing but the use of neomycin for egg dipping and for short term treatment of broilers prior to slaughter to reduce *Salmonella* infection is known to generate resistance in the *E. coli* population. There was a phase in the 1980s when layers were treated with an antibiotic on transfer to their laying accommodation followed by administration of a gut flora preparation to eliminate the increased susceptibility that follows therapy. This has largely been discontinued, mainly again as a result of the concern over inducing increased resistance.

Use of these antibiotics in the 1960s resulted in 1969 in the Swann Report which separated those chemotherapeutic agents which were to be used in human and veterinary medicine from those to be used for growth stimulation. The pharmaceutical industry was exceptionally innovative and developed a range of new antibiotics which generally targeted Gram-positive bacteria rather than the Gram-negative pathogens and which were not (at that time) used in medicine. Although some of these were highly effective at stimulating growth they did have the unfortunate effect of also stimulating colonisation and shedding of *Salmonella* by the birds. In addition, some of these drugs, particularly the glycopeptides, began to be used to treat MRSA in hospitals such that the division envisaged by Swann was beginning to break down. The Danish group of Fleming Bager, Frank Aarestrup and Henrik Wegener and others found that extensive use in poultry and pigs was generating glycopeptide resistance in *Enterococcus faecium* which was transposon-mediated to be transmissible. Resistant strains were able to colonise humans and found by Bill Noble to transfer the resistance to strains of *Staphyloccus aureus*. Extensive studies such as these led inevitably to the banning of growth promoters within the EU, starting in 1997 with avoparcin and completed in 2006. Although antibiotic use for growth-promotion continued in the US this is also now being re-examined.

Vaccines. In view of the above, vaccines seemed to be a more rational way forward. Not surprisingly, there was also some early work in the 1960s and 1970s, which was largely empirical, on the use of killed vaccines against the food-poisoning serovars although much work (largely in mice) showed them to be much less effective than live, attenuated vaccines as demonstrated by Smith for fowl typhoid in the 1950s. Killed vaccines were found to have some effect in reducing mortality but little effect on faecal shedding. A commercial vaccine, prepared by bacterial culture under conditions of iron starvation, has been shown to have some effect in the field, possibly as a result of the likely huge range of doses that birds will experience in the field with greater protective effect against the lower infectious doses. Although these remain in use and autologous vaccines retain a place, live vaccines have been shown by Willie Smith, Barrow and others to generate higher levels of protection in birds, the gold standard perhaps being that generated by a fully virulent strain. Although a number of effective candidate vaccines have been produced these are largely genetically manipulated and as such are unacceptable in the current climate. It is ironic that the most widely used live vaccine in Europe is antibiotic resistant and with undefined genetic lesions. The advantage of its extensive use is that there is no evidence for transmission to humans. Perhaps this situation will change and rationally attenuated vaccines will become more acceptable. The issue of cross protection between serovars remains a scientific problem to be solved although cross protection between serotypes within a serovar e.g. *S.* Gallinarum (via the 9R vaccine which was developed for fowl typhoid by Willie Smith in the 1950s) against *S.* Enteritidis was demonstrated by Barrow and has been exploited for a number of years.

During the past 25 years Barrow and his group showed that oral administration of live vaccines to newly-hatched chickens results in massive multiplication in the gut for a few days with a resulting competitive exclusion effect against related bacteria. This is thought to be largely a physiological/metabolic function, with heterophil infiltration into the gut mucosa also inhibiting invasion by *Salmonella* strains and other bacteria which infect during the following few days. These observations are not only interesting biologically but have some practical significance which has been explored by Methner, Richard Ducatelle and others and is utilised by some vaccine companies.

Competitive exclusion and probiotics. The use of competitive exclusion gut flora preparations began in 1974 with Esko Nurmi's publication which harks back to earlier work by Marjorie Bohnhoff and Philip Miller indicating the protective effect of the normal flora in mice. Nurmi's original findings, using a cultured intestinal flora, have been further developed to use caecal mucosal flora and commercial preparations have been developed by Nelson Cox, Norman Stern, Stan Bailey and others for a number of companies. Many countries are concerned over the use of undefined preparations which will contain many hundreds of different bacterial types, many of which will not be cultivable and for which quality control is difficult. However, attempts by Geoff Mead and Clive Impey to develop defined mixtures have been less successful, with lower levels of protection and the inability to protect other poultry (turkeys). The undefined preparations are highly effective, although more so experimentally than in the field, and have been used in many countries and extensively in Scandinavia

where they are an important part of the national *Salmonella* control programme.

The combination of withdrawal of growth promoting antibiotics and the desire to find single strains or simple combinations of strains has led to increased interest in probiotic organisms although the scientific basis for most of these is poor or unclear and most studies are empirical in nature. One area for future exploration is the development of probiotic organisms which have a rational basis for protection and which also have been found by Gabriel Perdigon and others to have immune-modulatory effects, stimulating innate immunity to provide short term protection against miscellaneous organisms and acting in an adjuvant capacity. Parallel studies on prebiotics, the use of chemicals or feed components which stimulate the colonisation by beneficial organisms, has also blossomed and studies on the rational combination of pre- and probiotics are highly desirable.

A holistic management approach to control. Despite our long interest in the biology of *Salmonella* and its interactions with other organisms and the host, most progress in reducing *Salmonella* prevalence in broiler, layer and breeder flocks has been made through an appreciation of the epidemiology of infection and a recognition that feed, the environment (housing) and the birds themselves are the main sources of infection for flocks. Although major outbreaks in Sweden in the 1950s led to the introduction of compulsory control measures there, it was not until the pandemic of a small number of phage types of *S.* Enteritidis in the late 1980s that the UK and the Netherlands, followed by the EU, introduced measures intended to control levels of *Salmonella* contamination in feed and to regularly monitor laying and breeding flocks for *S.* Enteritidis and *S.* Typhimrium, these being perceived as the two most important and virulent food-poisoning serovars. Monitoring has included the use of flock-level serology to identify flocks that did not require further bacteriological monitoring. Vaccination has also been accepted and used extensively in layers. This combination of measures has been very effective, with levels of infection in birds reduced to unprecedented figures of $<5\%$ in many parts of Europe.

These highly successful measures have had two consequences. One is that the two dominant serovars have been replaced in some countries by group C serovars, for which vaccines were not available. In addition, free trade resulted in the importation of poultry meat from countries where such measures have not been introduced and where use of antibiotics, including growth-promoters, is not regulated as they are in the EU. Not surprisingly, imported meat was found to contain more *Salmonella* and more antibiotic resistant organisms. Globalisation of food trade will inevitably introduce problems of this sort with economic or public health consequences or both.

New approaches. The future for *Salmonella* control will consist of the maintenance of high standards of management to prevent introduction and spread of infection and the continuous exploration of new approaches. Thus, novel ideas such as using lytic bacteriophages have been assessed experimentally by Barrow, Robert Atterbury and Ian Connerton, producing some reductions in levels of colonisation and remarkable effects on carcass decontamination. Whether such ideas will ever be applied remains to be seen but only by applying known successful measures and by exploring new ideas to counter new problems, will animal and public health problems, including those of *Salmonella* be tackled effectively.

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