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REVIEW ARTICLE

Pullorum disease and fowl typhoid—new thoughts on old diseases: a review

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Fowl typhoid and pullorum disease are two distinct septicaemic diseases largely specific to avian species and caused by *Salmonella* Gallinarum and *Salmonella* Pullorum, respectively. They were first described more than one century ago. Since their discovery, many efforts have been made to control and prevent their occurrence in commercial farming of birds. However, they remain a serious economic problem to livestock in countries where measures of control are not efficient or in those where the climatic conditions favour the environmental spread of these microorganisms. During the past 15 to 20 years there has been an explosion of genetic and immunological information on the biology of these two organisms, which is beginning to contribute to a better understanding of the organisms and their interaction with the host. However, it is not enough simply to understand the pathology in greater and greater detail. What is needed, in addition to this increase in basic knowledge, is creative thinking to challenge existing paradigms and to develop really novel approaches to infection control.

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Where are we now?

Fowl typhoid (FT) and pullorum disease (PD) are two distinct septicaemic diseases specific for avian species (disease of poultry and other production species including game birds, ducks and guinea fowl) that remain of major economic significance in many parts of the world. FT, caused by *Salmonella* Gallinarum, is an acute or chronic septicaemic disease that usually affects adult birds, although birds of all ages may be susceptible. PD, caused by *Salmonella* Pullorum, is an acute systemic disease more common in young birds. They were identified in the dying years of the nineteenth century and associated with endemic intermittent mortality or with outbreaks characterized by high mortality (up to 100%) and with reductions in productivity (Klein, 1889; Rettger, 1900). There has been a great deal of research activity on *Salmonella* organisms during the past 30 years, largely because of the ease of culture, genetic manipulation and sequencing and the opportunities offered by the mouse model for studying pathogenesis and immunity. In addition to *S.* Gallinarum and *S.* Pullorum, the zoonotic serovars have also been studied extensively in poultry. This has resulted in an unprecedented generation of genetic and immunological information on the biology of these two organisms with increasing interest in novel approaches to disease control.

A number of reviews of *S.* Gallinarum and *S.* Pullorum are available in the international literature (Shivaprasad, 2000; Lister & Barrow, 2008a, b; Shivaprasad & Barrow, 2008) and also in countries where they are major problems (Lee *et al.*, 2003, 2005; Hossain & Islam, 2004; Hossain *et al.*, 2006; Ahmed *et al.*, 2008; Pan *et al.*, 2009). It is not our intention to repeat this information, but rather to look at the new data that has been generated in the past 10 to 15 years and see how this has changed our view of these organisms and whether this information can change our approaches to controlling FT and PD.

How much of a problem are FT and PD worldwide? Is it possible to obtain accurate figures of incidence?

In many countries, official data relating to disease occurrence are poor because diseases are under-reported (many cases are likely to occur in backyard flocks) and the incidence of diseases such as FT and PD are in all probability gross underestimates. Although many countries are reported to be free of either FT or PD, this seems unlikely given the many wild avian species that can harbour these serovars. Thus, according to the updated World Organization for Animal Health database (OIE, 2010a, b), the USA reported its last case of FT in 1981; on the other hand, PD has been occurring in backyard flocks up until 2009 (USDA, 2009). This was a similar situation to the UK and several European countries that have apparently eliminated FT but find it more difficult to do so for PD, where the increasing amount of extensive free-range rearing with poor or no floor disinfection and the presence of various wildlife vectors (Davies & Wray, 1995a, b; Davies & Breslin, 2003) increases the risk of *Salmonella* infection (Auri *et al.*, 2010). However, although the UK regarded itself free of FT by 1986, *S.* Gallinarum was isolated from a commercial caged layer

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holding and in a small backyard flock in 2005. A third outbreak, geographically close to the original, was identified in a second commercial cage layer holding in spring 2006, and in autumn 2006 a fourth outbreak of FT was confirmed in a small free-range backyard flock of layer chickens. There was no confirmed connection between the backyard flocks and the commercial holdings (Defra, 2007). In contrast, cases of PD were reported from 1996 to 2004 mainly in free-range flocks but also in fancy breeds and game birds. FT has not been reported in Spain since 1991 and PD not since 1999. Poland reported its last occurrence of FT and PD in 1997. In contrast, France reported PD in 2004 and FT in 2005, and both were reported in Italy in 2005, 2006 and 2007. Both PD and FT have also been diagnosed in Germany between 2005 and 2008. Denmark has historically been free of FT for many years until outbreaks in the 1990s resulting from the illegal introduction of infected breeding stock into Germany followed by movement of contaminated crates into Denmark. Denmark also reported sporadic outbreaks of PD in ornamental, hobby and backyard birds in 2006, 2007 and 2008 (Dvfa, 2008). Russia reported 63 outbreaks of PD and 302 of FT between 2005 and 2008.

Although Mexico declared itself free of PD by 2002, outbreaks of FT occurred in 2005 and 2007. In Brazil, 10 outbreaks of PD occurred in 2004 and 82 outbreaks of FT were reported from 2005 to 2008. In Argentina, FT and PD were reported between 2005 and 2008.

Both diseases occur frequently in Asian poultry. From 1996 to 2008 FT was diagnosed in India, but the last outbreak of PD reportedly occurred in 2002. In China, more than 11,000 outbreaks of PD were reported between 2005 and 2008. In contrast, in Japan the last case of FT was in 1990 and of PD in 2002 (OIE, 2010a, b). Korea has taken a great interest in these diseases because, since the first case in the field in 1992, FT has spread throughout the country affecting mainly brown layers. Between 2000 and 2008, about 1000 FT outbreaks were reported. After adopting a nationwide vaccination programme, the prevalence of FT in Korea has decreased rapidly from 206 outbreaks in 2002 to 31 in 2008 (Kwon *et al*., 2010).

There are fewer reports of the situation in Africa. A FT outbreak was reported in commercial laying hens in Nigeria affecting 11,000 birds with the mortality rate ranging up to 25% (Ezema *et al*., 2009). In the same country, another 129 outbreaks of FT were diagnosed by the avian unit of a veterinary teaching hospital between 2003 and 2007 (Mbuko *et al*., 2009).

Based on available data it is clearly difficult to be precise about the occurrence and distribution in most countries, and most figures are obviously underestimates. It is clearly important to have some idea of the relative incidences of these infections since this affects the approach to control; that is, whether vaccination is used or test and slaughter is used. Ambient temperature with associated housing requirements and capacity for environmental control are likely to be important factors in incidence since these affect infection by wild birds and other vectors. In some countries there is public pressure towards free-range rearing for both broilers and layers. This also increases the risk of contamination from wild birds. The effect of ill-informed public pressure in promoting rearing regimens that are not beneficial to the health and welfare of birds is a new and difficult issue that adversely affects the incidence of several bacterial, viral and parasitic infections.

Is antibiotic resistance a problem?

Antimicrobial therapy is still being used for PD and FT in some countries. A variety of chemotherapeutic agents have been found to be effective at reducing mortality but are not able to eliminate infection from a flock since birds remain infected after chemotherapy has ceased and can be re-infected from the local environment (Moore, 1948; Wilson, 1956; Gordon & Tucker, 1957).

Extensive furazolidone used to control FT has led to a gradual increase in mutational resistance in *S*. Gallinarum, measurable *in vitro* by minimum inhibitory concentration (MIC) but not by gel diffusion, and which is also expressed *in vivo* (Smith *et al*., 1981). There has been an increase in resistance to fluoroquinolones from 0% to 6.5% (enrofloxacin) and to 82.6% (ofloxacin) amongst Korean strains (Lee *et al*., 2003) involving mutation in *gyrA* (Lee *et al*., 2004), in addition to resistance to a number of other antibiotics including ampicillin (13%), gentamicin (43%) and kanamycin (69.6%). Multi-resistance is also becoming more frequent. Class 1 integrons have been isolated more frequently from *S*. Gallinarum in Korea since 1996 (now 39%), with the risk that antibiotic resistance may become a structural part of these integrons enhancing transfer between strains (Kwon *et al*., 2002). In a study evaluating changes in antibiotic resistance patterns of *S*. Pullorum strains isolated between 1962 and 2007 from diseased chickens in China, high levels of resistance were found to ampicillin, carbenicillin, streptomycin, tetracycline, trimethoprim and sulphafurazole. An increase in multi-resistant strains between 2000 and 2007 was also observed suggesting that more rational use of antibiotic is desirable (Pan *et al*., 2009).

The widespread use of antimicrobial agents in poultry production has led to the occurrence of resistant bacterial species that can be transmitted to humans via the food chain (Tollefson & Miller, 2000) and is an international concern. Chemotherapy against any bacterial pathogen will have a selective effect on other bacterial species that are present in the animal that is being treated. *Escherichia coli* represents an enormous reservoir of resistance genes that are able to transmit resistance determinants readily to other species by conjugation. Practices such as spraying or dipping eggs with neomycin or gentamicin (Stuart & Keenum, 1970; Aziz *et al*., 1997) and short-term application of in-feed neomycin to reduce intestinal carriage of *Salmonella* by chickens has been found to induce increased resistance to *E. coli* in the chicken gut (Smith & Tucker, 1978).

How are *S.* Gallinarum and *S.* Pullorum taxonomically related to each other and to other serovars?

Salmonella enterica subsp. *enterica* serovars Gallinarum and Pullorum are no longer regarded as separate species (Grimont & Weill, 2007). Nowadays known as *S*. Gallinarum and *S*. Pullorum, they are indistinguishable by normal serotyping, belonging to serogroup D (both possessing O antigens 1, 9, 12 and non-flagellated: –,–) and are generally regarded as biotypes of the same serovar (Christensen *et al*., 1993). An early attempt

(Crichton & Old, 1990) to classify strains within this group of two serovars using a combination of biochemical markers (e.g. gas production, fermentation of dulcitol, maltose, rhamnose and xylose, and decarboxylation of ornithine) resulted in 94% of 86 strains falling clearly into three groups. There was little association between type and geographical occurrence. The 50 Gallinarum strains were fairly homogeneous, with one strain differing in dulcitol and decarboxylation of ornithine. In contrast, the 36 Pullorum strains, mostly from the UK, showed greater heterogeneity—with the majority of strains falling into two groups differing in rhamnose, xylose and gas production, with a small number of strains showing additional variations. A further small number of *S.* Pullorum strains showed characteristics associated with both *S.* Gallinarum and *S.* Pullorum; for instance, one of them was anaerogenic, fermented ducitol and did not decarboxylate ornithine, like *S.* Gallinarum; on the other hand, its maltose and rhamnose characteristics were not those expected of a *S.* Gallinarum strain. Meanwhile, another strain fermented dulcitol and maltose, like *S.* Gallinarum strains, but, like *S.* Pullorum strains, it was aerogenic and decarboxylated ornithine. The two *S.* Pullorum groups were also separated by Southern hybridization using a probe prepared from the type-1 accessory fimbrial gene (whether or not they were observed microscopically to be fimbriate both types were non-adhesive). The anaerogenic *S.* Pullorum type also correlated with phage type (mainly 2) and was widely distributed globally.

The presence of strains intermediate between *S.* Gallinarum and *S.* Pullorum as indicated above is supported by Porwollik *et al.* (2005) who used comparative genomics by microarray and found one *S.* Pullorum strain that appeared to be intermediate with *S.* Gallinarum in possessing *torRS* (TMAO reductase) and hydrolase, not possessed by most other strains of this biotype, but it lacked other regions of difference (ROD) normally associated with *S.* Gallinarum. This strain harboured three ROD that are all missing in the other common biovar Pullorum strains. It also lacked two ROD otherwise found only in strains of biovar Gallinarum. Based on the genetic profile, it may be more closely related to biovar Gallinarum than to biovar Pullorum (Porwollik *et al.*, 2005).

The serological relationship with other serogroup D serovars that also produce systemic disease, *S.* Enteritidis (murine typhoid, systemic disease in young and older immunologically compromised chickens) and *S.* Dublin (murine typhoid and diarrhoea with systemic disease in calves and systemic disease and carriage and abortion in immunologically compromised adult cattle), suggests a phylogenetic relationship. The use of multilocus enzyme electrophoresis and multilocus sequence typing together with increasingly available whole genome sequences for an increasing number of strains has supported this supposition.

Multilocus enzyme electrophoresis suggested that these two types were closely related but that they had evolved from a non-motile ancestor that was closely related to *S.* Enteritidis rather than to *S.* Dublin. Extensive multilocus enzyme electrophoresis analysis (Li *et al.*, 1993) revealed at least three lineages, namely *S.* Gallinarum, *S.* Pullorum and an intermediate lineage. There was again greater heterogeneity amongst *S.* Pullorum strains indicated by this and by sequencing of the *fli*C gene, which indicated that flagellin, if produced, would express the g and m and possibly other epitopes (Mortimer *et al.*, 2004). The true nature of this group of types will only be determined by analysis of a much larger number of strains.

The reason for non-motility and how this developed remains unclear. It has been suggested that the ancestor of these two types was *S.* Enteritidis-like with a broad host range that included birds. *S.* Gallinarum and *S.* Pullorum are thought to be monophyletic since they possess electrophoretic enzyme alleles that are unique or rare amongst other serovars, coupled with similar sized virulence plasmids and absence of intact 23S ribosomal RNA (Li *et al.*, 1993). The hypothetical scenario of evolution involved mutation from a *S.* Enteritidis-like ancestor to non-motility with accumulated neutral mutations. *S.* Dublin is thought to have evolved away from *S.* Enteritidis with the development of the g and p flagella epitopes but with minimal differences in enzyme genotypes (Porwollik *et al.*, 2005). In addition, a proportion of the globally distributed *S.* Dublin clone from North America and Europe is non-motile. Some of these strains are thought to be able to show motility (Selander *et al.*, 1992). The Vi antigen, unique to one clone of *S.* Dublin found in Europe and thought to be encoded by horizontally introduced genes, is not found in either the *S.* Enteritidis, *S.* Gallinarum or *S.* Pullorum clones (Porwollik *et al.*, 2005).

The occurrence of IS200, pulsed-field gel electrophoresis and ribotyping were used to compare strains identified by biochemical analysis as *S.* Pullorum or *S.* Gallinarum (Olsen *et al.*, 1996). Cluster analysis indicated a degree of homogeneity within each biotype but with a small number of atypical strains within each group and some strains that remained unclustered, indicating no correlation between phenotype and genotype as identified by pulsed-field gel electrophoresis. These genetic markers were also not well correlated with the differences in pathogenicity between the two biotypes. The IS200 patterns in these biotypes were also similar to those in different phage types of *S.* Enteritidis, also supporting a common heritage.

Comparative genome sequence analysis of *S.* Enteritidis and *S.* Gallinarum (Thomson *et al.*, 2008) supports the close taxonomic relationship between these two serovars and suggests that they may have split from a common ancestor by acquisition of a number of fimbrial genes, for example *lpf*, *pge* and *ste* (Clayton *et al.*, 2008), SPI-17 and other RODs with *S.* Gallinarum acquiring and losing genes different to *S.* Enteritidis. The analysis indicated a closer relationship between these two serovars than with *S.* Typhimurium (Thomson *et al.*, 2008). More genes are shared between *S.* Enteritidis and *S.* Gallinarum ($n = 188$) than between either of these and *S.* Typhimurium ($n = 39$ and $n = 16$, respectively). One of the features is the high similarity between *S.* Gallinarum and *S.* Enteritidis when compared with *S.* Typhimurium LT2, with average nucleotide identities of orthologs shared between *S.* Gallinarum and *S.* Enteritidis of 99.7% and 98.93% with LT2 (Thomson *et al.*, 2008).

Crosa *et al.* (1973) showed by DNA:DNA hybridization that, whereas *S.* Enteritidis and *S.* Gallinarum showed 95% and 91% hybridization to *S.* Typhimurium, respectively, the *S.* Pullorum strain studied showed 96%. Construction of genome restriction maps of *S.* Pullorum (Liu *et al.*, 2002) and *S.* Gallinarum (Wu *et al.*, 2005)

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indicated that, in comparison with other serovars such as *S.* Typhimurium, both exhibited two major inversions between *rrl*H and *rrl*G and between *his*A and *put*A. In comparison with *S.* Gallinarum, *S.* Pullorum has a further inversion between *rrl*D and *rrl*E. In *S.* Pullorum this gene order was observed in 12/16 strains studied. The hypothesis was developed that as a result of a large insertion (157kb) the chromosome had become unbalanced in terms of the position of the origin of replication (*ori*C) and the termination site (*ter*), and the various other changes that have taken place are an attempt in evolutionary terms to rebalance the genome.

Can the genomic information throw light on the basis of virulence and absence of motility?

Like other typhoid serovars, *S.* Gallinarum and *S.* Pullorum infect birds via the oral route where they invade via intestinal epithelial cells or lymphoid tissue localized mainly in the Peyer's patch and caecal tonsils. Infected phagocytes and free bacteria move to lymphoid tissues (liver, spleen, bone marrow), where bacterial multiplication takes place. They re-enter lymphoid tissue in the intestine by a completely unknown mechanism and are shed in the faeces. They colonize the gut poorly in the absence of clinical disease and rarely enter the human food chain.

Genomic analysis is beginning to reveal information that may contribute to our understanding of host adaptation and evolutionary aspects of pathogenicity.

The genome of *S.* Gallinarum is slightly smaller at 4659 Mbp compared with 4686 Mbp for *S.* Enteritidis but carries 309 pseudogenes compared with 113 in Enteritidis (204 and 25 in *S.* Typhi and *S.* Typhimurium, respectively) (Thomson *et al.*, 2008). It also has fewer tRNA genes ($n = 75$) than *S.* Enteritidis ($n = 84$) and is co-linear except for a single inversion of 817kb (about the rRNA operons) and a single translocation of a region (49 kb) located between two different rRNA operons. There are 130 coding sequences specific to *S.* Enteritidis in comparison with *S.* Gallinarum that either appear to be recent acquisitions with no evidence for them in *S.* Gallinarum or have been deleted from *S.* Gallinarum. These include ROD 14 and SPI-6 (now known to be a T6SS), which are degenerate in both serotypes (*S.* Gallinarum and *S.* Enteritidis). There is a much reduced prophage content compared with either *S.* Typhimurium or *S.* Enteritidis. The presence of a large number of pseudogenes in a strongly host-adapted bacterial pathogen is not new. Thus *Mycobacterium leprae* has more than 1000 pseudogenes when compared with *M. tuberculosis* (Cole *et al.*, 2001). The presence of a reduced genome indicates the relatively favourable environment within the phagosome of the infected macrophage and that fewer genes are required for systemic virulence than for the production of enteric disease. It is expected that the gene complement of the more host-adapted *S.* Pullorum will be even smaller. The absence of motility, at least, has been postulated to be an adaptation to the avian host (see below). The biological and molecular basis of host adaptation in any of these host specific serovars is not understood at all (Barrow *et al.*, 1994; Pascopella *et al.*, 1995; Steinbach *et al.*, 2000; Uzzau *et al.*, 2000, 2001; Paulin *et al.*, 2002; Eswarappa *et al.*, 2009; Osman *et al.*, 2009). Several studies indicate that for *S.* Gallinarum virulence and host specificity correlate with *in vivo* multiplication in the tissues but not with intestinal colonization or with invasion from the gut (Barrow *et al.*, 1994; Wallis *et al.*, 1999; Steinbach *et al.*, 2000; Chadfield *et al.*, 2003). The relatively high apparent rate of accumulation of mutations suggests a rapid rate of evolution associated with the host adaptation event, particularly in the development of *S.* Pullorum, suggesting that host adaptation may have developed independently in *S.* Gallinarum and *S.* Pullorum.

In the case of *S.* Gallinarum, several metabolic pathways have been lost, which must have a bearing on its intracellular lifestyle (Thomson *et al.*, 2008). These include the inability to use long chain maltodextrins, D-glucarate and hydrogenase 1. Importantly, there is an inability to utilize 1,2-propanediol through mutations in the *pdu* operons in addition to *ttr* (tetrathionate) and *cbi* (cobalamin), which are required for its oxidation. These are functional in *S.* Typhimurium and *S.* Enteritidis, which produce systemic disease in mice, but are not functional in *S.* Typhi (Parkhill *et al.*, 2001), which more closely resembles *S.* Gallinarum, suggesting that these two typhoid serovars have similar carbon source requirements in the intracellular environment that may relate to differences in these serovars or to differences in the host species or to both. The non-utilization of 1,2-propanediol may also, however, relate to the poor intestinal colonization ability in *S.* Gallinarum and *S.* Pullorum in addition to *S.* Typhi; recent unpublished evidence suggests that the 1,2-propanediol metabolism is important during colonization of the chicken intestine in *S.* Typhimurium (Barrow *et al.*, unpublished results).

S. Gallinarum also carries a mutation in *speC* encoding ornithine decarboxylase, making the one remaining intact arginine catabolic pathway, involving arginine decarboxylase, an essential biosynthetic route for putrescine (Thomson *et al.*, 2008). The mutation in *speC* could explain the inability of *S.* Gallinarum to decarboxylate ornithine, a defining feature of this *Salmonella* serovar (Crichton & Old, 1990).

Unlike other *Salmonella* serovars, *S.* Gallinarum and *S.* Pullorum are both unable to produce glycogen. In *S.* Gallinarum there are extensive mutations in *glgA, B* and *C*; *S.* Pullorum does not possess the same deletion in *glgC* but the nature of other mutations is currently unknown (McMeechan *et al.*, 2005).

The SPI3 genes *shd*A and *rat*B, associated with intestinal colonization, have also been lost functionally by *S.* Typhi and *S.* Gallinarum. Other genes associated with colonization and lost by these two serovars include those encoding the ability to use alternative electron acceptors dimethyl sulphoxide (*dms*A1, *dms*A2 in *S.* Gallinarum; and *dms*A2, *dms*B2 in *S.* Typhi) and trimethylamine *N*-oxide (*tor*S in *S.* Gallinarum; and *tor*R, *tor*C in *S.* Typhi) (Thomson *et al.*, 2008). The *bcs*G gene in *S.* Enteritidis contributes to biofilm production and extracellular survival. A mutation in *bcs*G in *S.* Gallinarum (Thomson *et al.*, 2008) might contribute to its poorer survival outside the host.

Although the virulence plasmid of *S.* Typhimurium does not appear to play a role in the initial intestinal phase of *S.* Typhimurium infection in mice, the plasmid of *S.* Gallinarum does contribute to the initial intestinal colonization, transient though it is, associated with the production of K88-like fimbriae in the same plasmid

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location as the *pef* locus in *S.* Enteritidis (Rychlik *et al.*, 1998).

S. Gallinarum and *S.* Pullorum are non-motile although there has been considerable speculation on the reversibility of this phenotype in *S.* Pullorum (Holt & Chaubal, 1997; Chaubal & Holt, 1999). The *fli*C gene in S. Gallinarum, responsible for flagellin expression, is entire. However, this serovar carries mutations in five genes (*cheM*, *flh*A, *flh*B, *flg*K and *flg*I) distributed in two loci involved in the synthesis of the flagellar structure. Why more mutations have not accumulated in more than five of the 50 genes responsible in *Salmonella* for motility remains to be seen. Flagellin, the main protein of the flagellar filament, is an important pathogen-associated molecular pattern responsible for alerting the host of early infection, in this case through stimulation of an inflammatory response via toll-like receptor 5 signalling following invasion. As a result of the absence of flagella, *S.* Gallinarum and *S.* Pullorum are able to invade from the alimentary tract without provoking a strong inflammatory response, perhaps favouring systemic infection and which might be a specific adaptation to avian hosts (Kaiser *et al.*, 2000; Iqbal *et al.*, 2005). Additionally, there is some evidence for the role of flagella in colonization and invasion in *S.* Typhimurium and *S.* Enteritidis (Dibb-Fuller & Woodward, 2000; Parker & Guard-Petter, 2001; van Asten *et al.*, 2004).

Genes such as *sop*A, *pip*B2 and *sif*B, which encodes TTSS effector proteins involved in intestinal inflammation and enteritis, are truncated in *S.* Gallinarum; an attempt to detect secreted SopA in this serovar was not successful (Thomson *et al.*, 2008). Rahman (2006) also found that SopB was not detectable in *S.* Gallinarum in contrast to most other serovars, but SopE could be detected (Rahman *et al.*, 2004, 2005). Other deleted genes that are involved in cellular interactions, including *big*A, have been lost in *S.* Gallinarum, suggesting that the enteropathogenic ability may be affected accordingly.

S. Enteritidis has an internal deletion of 24 kb within SPI-19, which *S.* Gallinarum does not have (Blondel *et al.*, 2009), presumably acquired since the split between these taxa. The *sopE* cassette, required for enteropathogenicity in several enteric serovars (Tsolis *et al*, 1999; Hopkins & Threlfall, 2004), is located in a cryptic lambda-like prophage in *S.* Gallinarum, *S.* Enteritidis, *S.* Dublin and also *S.* Hadar with similarity to the GIFSY-phages, indicating the capacity to spread and an indication of transfer into this group of serovars prior to their splitting into their existing serovars (Mirold *et al.*, 2001).

Of the 13 fimbrial operons possessed by *S.* Enteritidis, *std* is not present in *S.* Gallinarum (also reported by Porwollik *et al.*, 2005) and there are mutations in several genes of the other fimbriae, leaving only *fim*, *bcf*, *csg* and *ste* undisrupted (Thomson *et al.*, 2008). Both serovars carry fimbrial genes on the virulence plasmids. The five genes of the *pef* operon present in *S.* Enteritidis virulence plasmid are replaced by three encoding fimbrial genes in *S.* Gallinarum (see above) (Rychlik *et al.*, 1998; Thomson *et al.*, 2008).

Intracellular bacterial multiplication takes place, it is thought, largely through the activities of homologous/orthologous genes in *S.* Gallinarum that have been found to contribute to virulence in *S.* Typhimurium. These include SPI-2 genes whereas SPI-1 appears to contribute little to disease in poultry (Jones *et al.*, 2001). As with *S.* Typhimurium in mice (Shea *et al.*, 1999), SPI-2 does not appear to contribute to invasiveness after oral infection. SPI-2 also contributes to persistent infection in *S.* Pullorum, although how far this is simply a reflection of initial survival or not in macrophages rather than a specific association with long-term persistence is unclear (Wigley *et al.*, 2002). The exact manner in which the 19 SPIs, 13 fimbrial gene sets and several metabolic traits all combine to contribute to virulence and host adaptation of *S.* Gallinarum and *S.* Pullorum remains to be determined. *S.* Gallinarum also carries mutations in genes involved in drug resistance, protective responses and DNA restriction/modification (Thomson *et al.*, 2008). The virulence plasmid *spvRABCD* genes are present in both *S.* Gallinarum and *S.* Pullorum (Rychlik *et al.*, 2006) and are essential for clinical disease (Barrow *et al.*, 1987). Given the fact that so many chromosomal genes are known to contribute to systemic disease, it is tempting to regard the virulence plasmid genes as no more than a molecular switch with a close regulatory relationship to key chromosomal genes, possibly SPI-2 genes. Although it is essential for systemic disease, the virulence plasmid contributes little to host specificity since the plasmids of *S.* Gallinarum, *S.* Pullorum and *S.* Typhimurium appear to be fully interchangeable without affecting the virulence phenotype of the recipient strain (Barrow & Lovell, 1989).

Acute and chronic disease is also characterized by haemolysis and the presence of necrotic lesions in the heart and alimentary tract (Smith, 1955). These are correlated with bacterial multiplication (Christensen *et al.*, 1996). The cardiac lesions are characterized by myofibrillar necrosis with heterophils, lymphocytes and plasma cells being replaced by histiocytes (Shivaprasad & Barrow, 2008). They occur as disease progresses into a more chronic phase of infection both with *S.* Gallinarum and with *S.* Pullorum (Buxton, 1957); how far they are a response to bacterial antigens or a form of an autoimmune reaction is unclear. It is thought that they do not contain viable bacteria (Christensen & Barrow, unpublished data).

The genes *sly*A and *cly*A have been found to encode cytolysin production in *S.* Typhi and *S.* Paratyphi A (Libby *et al.*, 1994; von Rhein *et al*, 2009). The *cly*A gene was not detectable by PCR in 95 *S.* Gallinarum strains (Agrawal *et al.*, 2005). The *sly*A gene was detectable in 94 *S.* Gallinarum strains but its presence did not correlate with haemolytic activity (Agrawal *et al.*, 2005). The significance of these results remains to be seen. SlyA is an important regulatory component in a signalling cascade controlling SPI-2 gene expression in *S.* Typhimurium (Linehan *et al.*, 2005; Fass & Groismann, 2009; Yoon *et al.*, 2009).

Although the full genome sequence for *S.* Pullorum is not yet available, microarray studies comparing the gene content with *S.* Enteritidis indicate the absence of the *torRS* regulatory system, also found to be absent in *S.* Abortusovis but present in all other serovars, and a hydrolase also absent for a variety of other *Salmonella* strains. One *S.* Pullorum strain appeared to be intermediate with *S.* Gallinarum in possessing the *torRS* and hydrolase not possessed by most other strains of this biotype but lacked other RODs normally associated with *S.* Gallinarum (Porwollik *et al.*, 2005). A study using suppression subtractive hybridization has also identified a number of putative virulence genes in *S.* Pullorum that

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differ from *S.* Enteritidis and *S.* Gallinarum (Qiuchun *et al.*, 2009). These included *ipaJ* gene (an invasion plasmid antigen gene of *Shigella*), a gene for Colicin Y production (an antimicrobial protein encoded by an *E. coli* plasmid), fimbrial plasmid genes *faeH* and *faeI*, and *traG* that encodes a coupling protein with TraJ (involved in DNA translocation).

Is our current understanding of the epidemiology of FT and PD correct? What is the nature of vertical transmission and the carrier state?

S. Gallinarum produces FT in susceptible birds of all ages with variable to high mortality depending on the bacterial strain and genetic background of the host. In regions of a country where white egg and brown egg layers may be reared, clinical disease may be found only in the brown egg layers that are known to be more susceptible than white layers (Hutt & Crawford, 1960; Bumstead & Barrow, 1988, 1993; Berchieri, personal communication). Although outbreaks are reported in a wide variety of avian species (John-Brooks & Rhodes, 1923; Buxton, 1957; Pomeroy & Nagaraja, 1991; Shivaprasad, 2000; Shivaprasad & Barrow, 2008), the severity of the disease varies widely. Since the adoption of intensive breeding programmes, which started in the 1940s, susceptibility may have changed considerably over decades. For example, the main breed of duck reared in the UK is completely resistant to oral or parenteral infection with *S.* Gallinarum (Barrow *et al.*, 1999).

The difference in susceptibility between inbred resistant and susceptible lines of chicken to *S.* Gallinarum can be enormous with the median lethal dose by a parenteral route being <10 bacteria whereas in resistant lines it is $>10^8$ bacteria (Bumstead & Barrow, 1993). Similar but smaller differences were also observed with *S.* Pullorum infection in young birds, with differences between resistant and susceptible birds of between 100-fold and 1000-fold, similar to that seen with *S.* Enteritidis in birds of the same age. Moreover, among three commercial lines of light laying hens considered to be resistant to FT reared in Brazil, significant differences in the mortality rates were observed after experimental challenge with *S.* Gallinarum (Freitas Neto *et al.*, 2007). The indigenous naked neck skin chicken of Mexico also showed increased resistance to *S.* Gallinarum infection (Alvarez *et al.*, 2003). In chickens, the resistance to systemic salmonellosis appears to be expressed within cells of the macrophage–monocyte lineage (Wigley *et al.*, 2002) and the locus responsible for it is nominated *SAL1* (Mariani *et al.*, 1998). Other loci including *NRAMP1*, *TNC* and toll-like receptor 4 are also involved in the resistance, although their contribution is relatively minor and the MHC is not involved (Hu *et al.*, 1997; Mariani *et al.*, 1998; Leveque *et al.*, 2003). *SAL1* is located on chicken chromosome 5 (Mariani *et al.*, 2001). Polymorphisms of genes in these loci would explain the difference in susceptibility against systemic salmonellosis between different breeds and lines of chickens. Studies using outbred and local breeds also sometimes show characteristic differences in susceptibility to infection, which in some cases is thought to be associated with differences in heterophil numbers (Mdegela *et al.*, 2002; Msoffe *et al.*, 2006).

Although the oral median lethal dose of *S.* Gallinarum is approximately 10^4 colony-forming units, with the median lethal dose by a parenteral route being <10 bacteria (this representing a loss of 99.9% bacteria in the gastrointestinal tract), infection is also possible by the respiratory route (Basnet *et al.*, 2008). It is thought that uptake in the gut is via the surface lymphoid tissue since bacterial recovery after infection is greater from the Peyer's patch and caecal tonsil than from the secretory epithelium (Barrow *et al.*, 2000; Chadfield *et al.*, 2003). How *S.* Pullorum behaves during infection of newly hatched chicks following horizontal transmission after hatchery infection is unclear since in the gut of such birds with no gut flora even small bacterial numbers can multiply rapidly to reach densities in excess of 10^8 colony-forming units/g. In addition, immunity in birds at this age is extremely immature and therefore is not able to trigger a cellular immune response robust enough to avoid the systemic infection (Holt *et al.*, 1999). Therefore, in such highly susceptible individuals, higher mortality rates than those actually observed might be expected suggesting a degree of attenuation in *S.* Pullorum compared with *S.* Gallinarum, which is observed following parenteral inoculation (Barrow *et al.*, 1987; Barrow & Lovell, 1989). The *in vitro* down-regulation of interleukin (IL)-1β and IL-6 observed in experimental infection of epithelial cells (Kaiser *et al.*, 2000) is probably responsible for the absence of inflammatory exudates following oral infection (Henderson *et al.*, 1999).

Although the general dogma is that both *S.* Gallinarum and *S.* Pullorum are transmitted both vertically and horizontally, according to the older literature horizontal transmission is more significant for *S.* Gallinarum with both routes important for *S.* Pullorum (Beaudette, 1925, 1930; Beach & Davis, 1927; Hall *et al.*, 1949). Experimental work has shown that eggs produced by infected or reactor birds can be contaminated with *S.* Gallinarum (Gauger, 1937; Nobrega & Bueno, 1942; Jordan, 1956). However, there is less evidence for transmission of *S.* Gallinarum to progeny via the egg (Doyle, 1926; Beach & Davis, 1927; Moore, 1946). A series of studies recently attempted to reproduce persistent infection and vertical transmission with *S.* Pullorum and *S.* Gallinarum (Berchieri *et al.*, 2000, 2001). The *S.* Pullorum strain used by Berchieri *et al.* (2001) produced typical disease-free persistent infection of the liver and spleen following convalescence from early disease with resurgence of infection at onset of lay. Establishment of a persistent infection with *S.* Gallinarum was much more difficult. Depending on the dose, infection appeared to either induce clinical disease with mortality and eventual clearance from the tissues or no disease at all. Persistent infection was only possible with one inbred line of chickens (line N, which is a *Sal*I-resistant genotype) where persistent isolation from liver and spleen occurred up to 14 weeks post infection at 1 week of age.

S. Pullorum was shown experimentally to persist for more than 40 weeks and persistence occurred despite the presence of high-titre circulating specific antibody, suggesting an intracellular site of infection. This was shown to be within macrophages in the spleen (Wigley *et al.*, 2001). The slower clearance of *S.* Pullorum compared with *S.* Enteritidis from the spleen correlates with longer survival in splenic macrophages *in vitro* (Chappell *et al.*, 2009). Two possible explanations for the

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absence of immune clearance of *S.* Pullorum are either that infected macrophages are not visible to T cells within the infected bird, perhaps through down-regulation of MHC expression, or that the immune response itself is modulated during infection. The much lower levels of expression *in vivo* of Th1-associated cytokines interferon (IFN)-γ and IL-18 and higher levels of IL-4, frequently associated with a Th2-type response (Chappell *et al.*, 2009), suggests a degree of modulation by *S.* Pullorum towards a Th2-type response associated with high levels of antibody and poorer cell-mediated immunity. In clearance of *S.* Typhimurium from the tissues, there is an increase in T-cell proliferation with expression of IFN-γ and absence of IL-4 and IL-10 (Pie *et al.*, 1997; Beal *et al.*, 2004; Kogut *et al.*, 2005; Withanage *et al.*, 2005). The proposed scenario for *S.* Pullorum would not be unusual since a similar spectrum of immune activity is thought to occur in tuberculoid (predominantly Th1) and lepromatous (predominantly Th2) leprosy in man (Kaplan, 1993).

At the onset of sexual maturity, the bacterial numbers in the spleen of infected chickens increase and bacteria disseminate to other organs including the reproductive tract with resulting egg infection. Higher numbers of bacteria were isolated from the lower oviduct in comparison with the upper oviduct (Wigley *et al.*, 2005). Both male and female chickens develop a carrier state following infection but the increases in bacterial numbers and spread to the reproductive tract are phenomena restricted to hens, indicating that such changes are likely to be related to the onset of egg laying. The immunological responses during the carrier state and through the onset of laying in hens indicate that chickens produce both humoral and T-cell responses to infection, but at the onset of laying both the T-cell response to *Salmonella* and nonspecific responses to mitogenic stimulation fall sharply in both infected and uninfected birds. The fall in T-cell responsiveness coincides with the increase in numbers of *S.* Pullorum and its spread to the reproductive tract. Three weeks after the onset of egg laying, T-cell responsiveness begins to increase and bacterial numbers again decline. Specific antibody levels change little at the onset of laying but increase following the rise in bacterial numbers in a manner reminiscent of a secondary antibody response to re-challenge. These findings indicate that a non-specific suppression of cellular responses occurs at the onset of laying and plays a major role in the ability of *S.* Pullorum to infect the reproductive tract, leading to transmission to eggs. The loss of T-cell activity at the point of laying also has implications for *S.* Enteritidis infection and transmission to eggs, along with its control by vaccination offering a "window of opportunity" for pathogens in which infection may occur (Wigley *et al.*, 2005).

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Can our improved knowledge of these organisms be used to improve diagnosis?

A definitive diagnosis of FT and PD currently requires the isolation and identification of *S.* Gallinarum and *S.* Pullorum, although a presumptive diagnosis can be made from clinical signs, flock history, mortality and lesions. A positive serological reaction using the Rapid Slide Test can be useful in identifying infected birds as a flock test, although cross-reaction with other serogroup D serovars such as *S.* Enteritidis can obviously occur. To improve this, a specific ELISA may be used either in a laboratory or as a pen-side test with tap-washing and with reading the test by eye with appropriate controls. The ELISA has been used for both serovars using a combination of group D LPS and flagella antigen to eliminate positive reactions caused by *S.* Enteritidis infection (Barrow *et al.*, 1992a; Berchieri *et al.*, 1995).

Molecular methods are being sought to differentiate these two serovars. The *fliC* gene present in *S.* Gallinarum and *S.* Pullorum has polymorphisms at codons 316 and 339 (Kwon *et al.*, 2000). These polymorphisms were confirmed by sequencing in 41 strains of *S.* Pullorum and 52 *S.* Gallinarum, and a polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) using *HinP1I* was used to differentiate these serovars. The *rfbS* gene also has polymorphic nucleotides at positions 598 and 237 specific for *S.* Gallinarum and *S.* Pullorum, allowing the differentiation of these two serovars using PCR-RFLP for this gene (Park *et al.*, 2001). The *rfbS* polymorphism was also used for development of a one-step allele-specific PCR method capable of distinguishing *S.* Gallinarum from others (Shah *et al.*, 2005). A PCR-RFLP, using as a target the gene *speC* (gene associated with ornithine metabolism) and the enzyme *Eco*RI, was also able to differentiate *S.* Pullorum from *S.* Gallinarum strains (Ribeiro *et al.*, 2009). Recently, duplex PCR primers have been designed to target polymorphic regions of the genes *glgC* and *speC* in *S.* Gallinarum and could be used to differentiate these two serovars. The duplex PCR assay was validated in 53 *S.* Gallinarum and in 21 *S.* Pullorum strains previously typed (Kang *et al.*, 2010).

The publication of the whole *S.* Pullorum sequence would allow the identification of other regions of difference between these two serovars that could be used as alternative genetic markers for differentiation.

Do we have any novel ideas and approaches to infection control?

Control of these infections has depended on the level of infection in the region. Serological testing and slaughter is used where levels of infection are low and/or where eradication can be contemplated, while vaccines are considered where levels of infection are high or where eradication is not an option, for example where environmental control is impossible. Chemotherapy is also used with some long-term harmful effects (see above).

Early work indicated that live vaccines were much more effective and protective than inactivated vaccines, although some inactivated vaccines are currently produced and used for controlling *Salmonella* infection (Okamura *et al.*, 2007). The first effective live, attenuated vaccines 9R and 9S (Smith, 1956) were produced decades ago; and although 9S was more protective than 9R, the latter did not induce the production of lipopolysaccharide-specific circulating antibodies and therefore did not interfere with the whole blood agglutination test.

Since that time a long list of attenuated strains has been studied as candidate vaccines, some of which additionally are rough, and thus do not induce interfering antibodies, but some produce less than effective protection and few are as protective as the 9R vaccine.

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Those that are less protective include a rough virulence plasmid-cured derivative (Barrow, 1990), an *aroA–serC* (Barrow *et al.*, 2000) and an undefined rough mutant (Purchase *et al.*, 2008). More protective mutants include *phoA* (Barrow *et al.*, 1992b), *aroA* (Griffin & Barrow, 1993), *nuoG* (Zhang-Barber *et al.*, 1998), *crp* (Rosu *et al.*, 2007) and a *cobS–cbiA* double mutant (Penha Filho *et al.*, 2010), but none of these is rough.

One advantage of the 9R, and presumably of other vaccines, is that it is additionally protective against *S.* Enteritidis (Barrow *et al.*, 1991; Feberwee *et al.*, 2001). The reverse is also true with the Lohmann Animal Health AviPro Salmonella Vac E (*S.* Enteritidis vaccine) protecting against fowl typhoid (Chacana & Terzolo, 2006). The 9R vaccine has been used extensively (Gordon & Luke, 1959; Smith, 1969; Silva *et al.*, 1981a; Lee *et al.*, 2007) but retains some virulence for newly hatched and young chickens (Lee *et al.*, 2005) and persists in the tissues for several weeks (Barrow *et al.*, 1991).

Work by a number of groups over the years has indicated that the stimulation of innate immunity by the presence of live bacteria, either systemically (Mackaness, 1964; Blanden *et al.*, 1966) or in the intestine (Foster *et al.*, 2003), induces a rapid and profound resistance to infection. In addition to using live vaccines, modulation of the innate response has been done also by the administration of immune lymphokines harvested from *S.* Enteritidis–immunized chickens. In this case, administration of lymphokines, harvested from cultures of concanavalin-stimulated T cells obtained from the immunized birds, to chicks in contact with *S.* Gallinarum-infected birds (seeders) reduced mortality in contact chicks (from 69% to 15%) but had little effect on the percentage isolation from the tissues (from 83% to 61%) (Lowry *et al.*, 1999). This approach has been used to increase resistance of 1-day-old chicks to invasion by *S.* Enteritidis (Ziprin *et al.*, 1996; Kogut *et al.*, 1998) and *S.* Gallinarum (Kogut *et al.*, 1996) when administered to the birds that were subsequently challenged. The lymphokines are known to have immunomodulatory effects including stimulation and activation of heterophils.

Administration of live *Salmonella* vaccines by the oral route can also induce resistance to colonization. This is by a competitive colonization (competitive exclusion) where the protective strain occupies the physical or metabolic niche normally occupied by the challenge strain. Thus, intestinal colonization by *S.* Enteritidis (Chacana & Terzolo, 2006) or even by the unrelated *S.* Infantis (Barrow *et al.*, unpublished data) has been shown to reduce significantly the mortality that follows challenge with *S.* Gallinarum. The use of gut flora preparations for classic competitive exclusion has had a less than satisfactory effect on *S.* Gallinarum in comparison with controlling colonization by *S.* Enteritidis or *S.* Typhimurium. Protection can be demonstrated in birds of no more than a few days old whereas challenge in birds when older or through contact resulted in poorer protection (Nisbet *et al.*, 1998). It was considered not to be a practical approach to control FT (Silva *et al.*, 1981b), possibly because *S.* Gallinarum relies less on extensive colonization for infection and is normally able to infect birds with a mature gut flora. It has been suggested that the disappearance of *S.* Gallinarum and *S.* Pullorum from European and US poultry flocks during the period from the 1960s to the 1980s has been responsible for the resurgence of selected phage types of *S.* Enteritidis. It was postulated that the increase in human cases of salmonellosis caused by *S.* Enteritidis was triggered by this serovar filling the ecological niche vacated by *S.* Gallinarum and *S.* Pullorum in poultry flocks. *S.* Enteritidis became established in poultry flocks in the 1960s, which coincided with the eradication of *S.* Gallinarum and *S.* Pullorum from domestic fowl. Since these three pathogens share common surface antigens (O 1, 9 and 12), it is suggested that the flock immunity generated by the two avian *Salmonella* biotypes prevented *S.* Enteritidis from circulating in poultry flocks in the first half of this century (Bäumler *et al.*, 2000; Kingsley & Bäumler, 2000). How realistic this scenario is at the level of populations and individual birds is uncertain.

Other biological approaches have been considered for controlling spread of *S.* Gallinarum, including probiotic preparations, which have not so far been shown to have a significant effect against the pathogen *in vivo* (Audisio *et al.*, 1999; Gusils *et al.*, 1999a, 1999b). Many probiotic preparations contain high numbers of lactobacilli that normally produce large quantities of volatile fatty acids such as formic acid. The incorporation of these into feed has been shown to inhibit gut colonization by zoonotic serovars of *Salmonella* (Iba & Berchieri, 1995; Van Immerseel *et al.*, 2005, 2006; Sterzo *et al.*, 2007). One study also showed that in-feed formic acid was able to reduce clinical fowl typhoid from 76% to 33% following contact spread (Berchieri & Barrow, 1996).

Concluding remarks

Whole-genome sequencing of pathogens and host in addition to the post-genomic technologies that develop from this, such as transcriptional analysis at the level of the genome and increased availability of immunological markers for livestock species, have begun to change the study of bacterial infections in the past few years. In the case of *S.* Gallinarum and *S.* Pullorum, the scientific community is a long way from fulfilling the potential for exploiting this sort of information. The *S.* Gallinarum genome sequence has enabled us to link certain virulence phenotypes to individual genes. The genome sequence is not yet available for *S.* Pullorum but soon will be. The availability of high-throughput sequencing will shortly enable sequencing of whole bacterial populations, enabling a much more comprehensive view of bacterial evolution amongst related bacterial species. The chicken genome will increase reagent availability and facilitate a greater understanding of the immune response and particularly the carrier state. The possibility of immune modulation to reduce tissue carriage for these organisms can then be considered.

Comparisons of complete genome sequences also facilitate the development of improved molecular identification and diagnostic tools. The advent of molecular pen-side tests is perhaps not too far into the future.

Availability of sequences also enables a wider range of attenuations for live vaccine design such that the correct combination of immunogenicity on the one hand, and virulence and attenuation on the other, together with engineered serological markers is now possible. The issue of the use of genetically-modified vaccines in the human food chain nevertheless requires rational discussion.

As with many livestock diseases, including tuberculosis and brucellosis, control measures have been worked out many years ago. In the case of PD and FT, the limitations to housing and environmental management in many countries, where contact with the environment cannot be avoided, suggest that different approaches involving biological interventions may be necessary. This is also true in those countries where a method of rearing perceived by the public to be healthier (i.e. extensive, free range) involves greater contact with environmental sources of infection. It is in these situations that live vaccines, nutritional (short chain fatty acids) interventions and other novel approaches must be considered, either individually or in combination.

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