ELISAs and the serological analysis of salmonella infections in poultry: a review

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Large increases in the number of cases of human food-poisoning caused by salmonella have occurred in several countries in recent years. In England and Wales the annual number of bacteriologically confirmed cases rose from 10665 in 1981 to 30112 in 1990 and it is generally accepted that these figures are underestimates. The reasons for the unprecedented increase are largely unknown but may include increases in the consumption of convenience foods, poultry, and poultry products, together with a dramatic increase in *Salmonella enteritidis* infections in poultry. *S. enteritidis* and *S. typhimurium* are now the predominant serotypes both in human disease and in poultry.

The public and professional interest in *S. enteritidis* and other salmonellas has created an incentive to produce accurate data on the prevalence of salmonella-infected flocks and of salmonella-infected chickens within flocks. Bacteriological methods, are required by recent legislation [1, 2], and have been the traditional means of obtaining such data. However, serological methods can be used to detect infection with a number of serotypes. In the UK, the two serotypes most frequently isolated from poultry, and which produce most disease in man, *S. typhimurium* and *S. enteritidis*, are invasive for poultry. Other invasive Salmonellae also include those designated *Salmonella arizona* and *S. gallinarum* and its biovar pullorum. Oral infection with these organisms normally leads to the production of circulating antibody mainly on the IgG class. A major advantage of serological over bacteriological methods is that whereas salmonella organisms are excreted intermittently [3] serum IgG concentrations are persistent. The logistical problems of sampling large numbers of birds to detect low levels of infection within a flock can thus be reduced. One major disadvantage is that soon after infection serum IgG concentrations will be low (although increasing) whereas bacterial excretion will be at a maximum. Also, serotypes generally regarded as non-invasive, although perhaps less numerically significant from the public health point of view, may not be detected serologically. Such tests are therefore probably only applicable for initial screening of poultry for invasive serotypes such as those mentioned above.

A number of serological tests have been developed for detecting invasive serotypes, the most successful being slide agglutination using either serum or whole blood for the detection of poultry flocks infected with *S. gallinarum* or its biovar pullorum [4, 5]. This test has been applied successfully for more than 50

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years and has contributed considerably to the control of pullorum disease and fowl typhoid from flocks in several countries. The test is, however, crude and has been found to be too unreliable and insensitive for use with other serotypes [6]. Tube- and microagglutination tests and the more sensitive microantiglobulin tests have been applied to experimental and field infections with serogroups B, C and D. However, these tests are cumbersome and do not lend themselves readily to extensive use for large scale flock screening.

The use of ELISAs

Recent advances in ELISA (enzyme-linked immunosorbent assay) technology, including miniaturization and mechanization together with availability of commercially-prepared high quality reagents, has resulted in the application of the technique to the detection of infection caused by several avian pathogens such as infectious bronchitis virus [7], turkey rhinotracheitis virus [8], *Pasteurella multocida* [9] and *P. anatipestifer* [10]. ELISAs based on lipopolysaccharide and Vi antigens have been used to detect *S. typhimurium* and *S. typhi* infections in rodents and man respectively [11, 12, 13]. Nagaraja and colleagues [14, 15] used an ELISA for the detection of *S. arizona* in turkey flocks in the USA. However, it is only recently that the assay has been applied to *S. typhimurium*, *S. enteritidis* and other serotypes of major public health concern [16].

The ELISAs for avian salmonellas are indirect, detecting primarily specific IgG in serum or IgG in egg yolk, although the assay can also be adapted to detect specific IgM produced early in infection [17]. However, the nature of the IgM response following experimental salmonella infection is unclear. Using commercial reagents, IgM was not detected in commercial chickens naturally infected with *S. enteritidis* [18] although the time of infection of these birds would not be known. Neither IgM or IgG were detected in commercial layers experimentally infected with *S. enteritidis* PT4 [19]. The apparent absence of detectable IgM by ELISA was puzzling since it contributes considerably to the tube agglutination reaction [20]. However, later studies by these authors indicated that considerable quantities of specific IgM are detectable apparently for many weeks after infection. In these cases the concentrations measured were often greater than those of specific IgG [21, 22].

The detecting antigen used by most workers and which has considerable discriminatory power is lipopolysaccharide (LPS), prepared from a particular serotype or from another from the same serogroup. Cross reactions can occur because of common antigens possessed by different serotypes. Flagella antigens are also highly discriminatory but have not yet been tested as extensively on field samples as has LPS. Other antigens of varying specificity have been used largely for preliminary screening purposes although some claims for specificity have been made. They include surface protein antigens, some of which are said to be specific for *S. enteritidis* [14, 15, 23, 24], sonicated whole-cell soluble protein antigen [17] and a heat-extracted antigen [20]. The use of such antigens results in more birds being designated as infected than when LPS is used as the antigen. It is thought that such results indicate false positive reactions although whether this is so is unclear.

Most work has involved salmonellosis in chickens although the assay has been applied to turkeys [14, 15] and ducks (Hatfield et al., unpublished findings).

Experimental salmonellosis in chickens

Work with experimental salmonella infections assists in understanding the parameters which may affect the capacity of the ELISA to detect such infections. Experimental work, involving experimental *S. typhimurium* infection in specified pathogen-free (SPF) chickens [25] indicated that varying quantities of specific antibody of all three Ig isotypes were found in serum, bile and intestinal washings.

High IgG titres persist for many weeks following infection with *S. enteritidis* [23, 26], *S. gallinarum* [27] and *S. typhimurium* [28] respectively. In the last case high titres were still present 45 weeks after infection. Thus individual chickens may cease to excrete salmonella organisms while IgG titres persist. The unreliability of cloacal swabbing as an indicator of flock infection is generally accepted. However, elevated salmonella-specific IgG titres in the absence of detectable faecal excretion [17] may indicate current infection either in chickens other than those which have been bled or may suggest the presence of salmonella in the house environment.

Reinfection of chickens with the same organism does not seem to boost the serum IgG titres greatly [3, 25, 27]. The IgG titre obtained is, to some extent, dose dependent and is related to the extent of faecal excretion but not necessarily so for individual birds [21, 28]. This again emphasizes the value of the ELISA and presumably of other quantitative serological tests, as a flock rather than an individual bird test, since the intermediate IgG titres obtained after infection with a small number of salmonella organisms may be difficult to interpret.

Because of its high IgG concentrations egg-yolk [29, 30] rather than serum has already been applied to other avian pathogens such as Newcastle disease virus, infectious bronchitis virus [31] and mycoplasma [31, 32]. It has been applied to *S. enteritidis, typhimurium* and *gallinarum* [24, 27, 28, 33, 34]. In chickens experimentally infected with either *S. typhimurium* or *S. gallinarum*, specific-IgG titres in egg yolk were found (after an initial lag) to be very similar to serum titres [27, 28].

LPS is the most frequently used discriminating detecting antigen. Sera obtained from chickens infected either with *S. typhimurium* [28] or with *S. gallinarum* [27] yielded much higher titres with homologous rather than heterologous LPS as detecting antigen. LPS from serogroups B, C_1 and C_2, D and O-35 were tested. Similar specificity can be demonstrated using group B and D LPS tested with sera raised in chickens against salmonella strains from different serogroups such as B, C_1, C_4, D, E_1, E_4 and O-35 [17, 18]. Differentiation between infections caused by *S. enteritidis, S. gallinarum* and its biovar pullorum using different LPS preparations as coating antigens has also been claimed [35] but there have been no further reports of this. However, varying degrees of cross reactions between group B and D LPS have been demonstrated with sera from chickens infected with *S. typhimurium* [20, 27, 28]. This cross reaction has also been demonstrated by immunoblotting [18]. Chart and colleagues [18] found that antibodies against the 12 antigen were responsible for this.

Experimental infection of chickens with other members of the Enterobacteriaceae does not induce the production of high titres of cross-reacting antibodies [20, 27, 28]. Organisms tested include avian pathogenic *E. coli* serotypes, and strains of citrobacter, klebsiella and proteus.

The little experimental evidence available indicates that the antibody response to LPS antigen in chickens infected with a rough salmonella strain is poor [28; Barrow, unpublished observations]. The public health significance of this will have to be assessed. Flagella-specific IgG antibody would still be detectable. However, recent work has indicated that immunization with some smooth, live, attenuated salmonella vaccines might also generate only low LPS-specific IgG titres. Thus, concern over incompatibility between the use of some live vaccines and LPS-based ELISAs might prove to be exaggerated.

The use of flagella antigens would allow differentiation between infections caused by serotypes possessing the same LPS structure, and has particular value in differentiating infections caused by S. enteritidis from those caused by the non-flagellate S. gallinarum and its biovar pullorum. Humphrey and colleagues [19] and Chart and colleagues [18] were unable to detect flagella (gm-H)-specific IgG or IgM in chickens infected either experimentally or naturally with S. enteritidis. In contrast, others have detected strong flagella responses in chickens infected either with S. enteritidis or S. typhimurium [25, 27, 28, 36]. However, one interesting feature found by Timoney and colleagues [36] is that not all chickens experimentally infected with S. enteritidis develop high antibody titres; in the majority of the birds no raised titres were observed.

With a serotype such as S. typhimurium the extent of invasiveness appears to have little effect on the IgG titres generated [17]. However, the extent of multiplication of the bacteria in the tissues did appear to affect the immune response. Bumstead and Barrow [37] found that inbred lines of chickens differed in their susceptibility to S. typhimurium because of the varying ability of the inoculated strain to multiply in the reticuloendothelial system. The LPS-specific IgG response varied considerably in these lines; the more susceptible lines generated the highest titres [28]. There is also some suggestion that non-specific factors, including handling the chickens [28] and the onset of lay [27], may affect the IgG response.

The ELISA is relatively robust and can accommodate poor quality sera. Antibody titres do not deteriorate if serum or blood is dried on to absorbent paper [17, 38]. Dried serum can be stored at room temperature for several weeks without a loss of titre. Serum dried on paper can be treated with some disinfectants such as phenol vapour or chloroform with no effect on antibody titre. However, ethylene oxide, formalin vapour and γ-irradiation all reduce titres to varying degrees [17]. Heat inactivation of serum (56 °C, 30 min) has no effect on antibody titre.

Application to field infection

Several ELISAs have been used to study field infections by S. enteritidis, S. gallinarum and its biovar pullorum. Little work has been reported on its use in studying S. typhimurium infections. Both serum and egg-yolk have been used as samples. In most cases the logistics of flock sampling require that the absorbance value obtained with a single serum dilution rather than titration is used as an estimation of antibody concentration. Cooper and colleagues [35] found high group B LPS-specific titres ($\log_2$ 7·8–13·6) in 25 sera taken from 3 laying and breeder flocks infected with S. enteritidis. In contrast only 1 of 10 sera taken from a flock infected with S. typhimurium showed a high titre ($\log_2$ 8·96) when tested

against group D LPS. The cut-off point used to decide whether an absorbance indicated infection was based on OD values obtained by testing sera from uninfected SPF chickens but sera from no other salmonella-free chickens were examined.

Using the same ELISA Nicholas and Cullen [20] found high absorbance values, determined with LPS and heat-extract antigen as detecting antigen, with 25/40 and 40/40 sera respectively from a flock of layers, 10/40 of which were identified bacteriologically as infected. Absorbance values obtained with 40 sera from a *S. enteritidis*-free flock were all less than the threshold value.

Chart and colleagues [18] examined 29 laying hens, 10 of which were found to harbour *S. enteritidis*. A wide range of absorbance values were obtained with sera from these birds. No attempt was made to classify chickens as infected or not on the basis of the ELISA results. A second study involved six different flocks all of which had been shown to be infected with *S. enteritidis* [39]. Absorbance values from 99 sera from one flock were very low, perhaps because of a recent infection. From the remaining five flocks a wide range of absorbance values were obtained in each case, the authors concluding that *S. enteritidis*-infected flocks probably vary considerably in the levels of LPS-specific IgG detectable by their ELISA.

Using a flagella antigen prepared from a *S. enteritidis* strain in an ELISA, Timoney and colleagues [36] examined a *S. enteritidis*-infected breeder flock and un uninfected layer flock. Of 151 serum samples from the uninfected flock, all produced absorbance values of less than 0·5 and 147 were less than 0·25. Of the 47 sera from the infected flock 32 were greater than 0·50 and 14 were greater than 1·0.

Fewer data are available from testing egg yolk for *S. enteritidis* specific IgG. Dadrast and colleagues [24] indicated in a pilot study that 60% of egg yolks from an infected flock had high LPS-specific IgG titres. Nicholas and Andrews [34] studied three flocks infected with *S. enteritidis* and one infected with *S. typhimurium*. The infection rates in the first three flocks determined by ELISA with group-D LPS as detecting antigen and with a threshold determined with SPF sera were 20/100, 99/180 and 55/180. Using a heat-extract antigen the rates were higher. None of the yolks from the *S. typhimurium*-infected flock was considered infected using *S. enteritidis*-derived detecting antigens in the ELISA. When *S. typhimurium*-derived antigens were used the infection rates in this latter flock were considered to be 0/29 with egg yolk and 6/29 with sera. McLeod and Barrow [40] looked at egg yolks produced from chickens in 17 laying houses owned by two companies. *S. enteritidis* was isolated from four houses from which were obtained eggs yielding high absorbance values. From these houses 159/300 eggs had absorbance values of greater than 0·2 and 58/300 greater than 0·8. In contrast 177/450 eggs taken from the 13 uninfected houses produced values of less than 0·1, 416/450 less than 0·2 and all 450 less than 0·4.

Barrow and colleagues [41] examined sera from seven flocks, four of which had bacteriological evidence of *S. typhimurium* infection. High antibody titres detected by the whole-cell soluble protein sonicate antigen were found in three of these flocks and in none of the three flocks considered uninfected. Nicholas and Andrews [34] found that 6/29 egg yolk samples from a flock of free-range layers from which *S. typhimurium* had previously been isolated were deemed to be positive by ELISA using group B LPS and one was positive using group D LPS.

Timoney and colleagues [36] used flagella prepared from *S. enteritidis* to

differentiate field infections caused by this serotype from those caused by *S. gallinarum* biovar pullorum, since all three serotypes mentioned above belong to group D (O-1, 9, 12). All of 8 sera from a breeder flock and 10 from a layer flock, both infected with pullorum, produced absorbance values of less than 0·25, a similar profile to an uninfected flock examined simultaneously. In contrast, high absorbance values of greater than 0·5 were seen in over 40 % of chickens either experimentally or naturally infected with *S. enteritidis*. Barrow and colleagues [27] examined four Brazilian flocks one of which showed evidence of fowl typhoid. Sera from the three uninfected flocks gave relatively low absorbance values when tested with group D LPS as detecting antigen (all less than 0·35 with the exception of one at 0·6) whereas 75 % of the sera from the infected flock gave absorbances greater than 1·0. Absorbance values obtained with flagella prepared from *S. enteritidis* (g, m-H) were uniformly low.

Comparison between the performance of ELISA and other tests

Several comparisons between the performance of the ELISA and other serological tests have been made. In all the ELISA was apparently more sensitive and identified more infected individual birds than did the agglutination tests, including slide agglutination. Cooper and colleagues [35] found this for chickens taken from three flocks infected with *S. enteritidis* and one infected with *S. typhimurium*. The one chicken in the latter flock which gave a high absorbance value with the ELISA was not identified by any of the other tests, including the macroantiglobulin assay. Nicholas and Cullen [20] found that all of 40 serum samples obtained from a *S. enteritidis* infected flock were deemed positive with heat-extract antigen as detecting antigen, 25 positive by LPS-ELISA, 39 positive by microantiglobulin test, 9 by whole blood agglutination test and 3 by rapid slide test and by microagglutination. In addition to quantitative differences between the tests for individual sera, these authors found that high absorbances and titres detected by ELISA and the macroantiglobulin assay respectively persisted in contrast to the titres from other tests which fell away more quickly. This was ascribed to the fact that agglutination tests tend to detect IgM rather than IgG. In a further study of *S. enteritidis*-infected flocks [34], using egg yolk, the rapid slide test identified consistently fewer chickens than did the ELISA. In a study of *S. arizona* and *S. enteritidis*-infected turkeys [14, 22] the ELISA and microantiglobulin tests produced more positive results and for a longer period of time than either slide agglutination or microagglutination. Good correlation between ELISA and slide agglutination reactions was found for sera obtained from chickens infected with *S. gallinarum* [28, 38].

Chart and colleagues [18] found reasonably good correlation was obtained between the absorbance values obtained with the ELISA and the qualitative results obtained by slide agglutination and immunoblotting for sera from a flock infected with *S. enteritidis*. However, exceptions were observed in that some non-agglutinating sera produced reasonably high absorbance values while two agglutinating sera produced low absorbances. Further differences between the results produced by slide agglutination and ELISA were found in a subsequent study [39] where in five flocks infected with *S. enteritidis* a poor correlation was found for individual sera, although high and low absorbances for the ELISA and positive and negative agglutinations were found in each of the five flocks. The

relative merits of the ELISA and slide agglutination in detecting *S. enteritidis*-infected flocks using either sera or lysed blood [42] have recently been discussed extensively in correspondence [43–46]. Previous work which might be of relevance to this discussion is the finding that over the years some authors find slide agglutination to be unreliable and insensitive for *S. typhimurium* [6].

The above discussion indicates that, as for other serological tests, the ELISA must be regarded as a flock test and confirmatory bacteriological investigations should be carried out. As a flock test carried out at intervals, it should be at least as sensitive as bacteriological procedures which must be limited by the relatively small number of samples specified by current legislation [1, 2]. Some of the discrepancies between different ELISA tests described above are probably the results of the use of different systems by different laboratories. Interpretation of results produced by different groups would be more meaningful if the different ELISAs currently used were standardized as recommended recently [16]. Before full field sampling trials are carried out the limitations of each assay should be assessed under controlled conditions by studying experimental infections. Another possible cause of discrepancy is the calculation of the base line (cut-off-values) from SPF chickens by some laboratories, and from commercial chickens by other groups. Whereas the absorbance values obtained from uninfected SPF birds are invariably low [20, 35], those obtained from uninfected commercially reared birds may be higher [36, 40]. The apparent absence of infection in these commercial chickens was probably real because the spectrum of absorbance values was completely different to that obtained from infected birds. All these problems need to be solved before the ELISA is acceptable to the animal health authorities as an adjunct to bacteriological procedures.

On the basis of the results obtained in experimental work the ELISA appears to satisfy most of the requirements for its adoption as a serological test to detect infections produced by *S. typhimurium*, *S. enteritidis*, *S. gallinarum* and its biovar pullorum and other invasive serotypes. It appears to be among the most sensitive of the currently available tests and is easier to carry out than many tests. It allows for quantitation of antibody and allows discrimination between some serotypes and between infections caused by salmonella and by other bacteria.

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