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Further observations on the serological response to experimental *Salmonella typhimurium* in chickens measured by ELISA

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SUMMARY

An indirect ELISA developed for the serological detection of *Salmonella typhimurium* in chickens using lipopolysaccharide as detecting antigen has been evaluated further in experimental infections. Following oral infection of 24-week-old laying hens with an invasive strain of *S. typhimurium*, high titres of specific circulating IgG were induced which were maintained for 20 weeks. Similar IgG titres were found in egg yolk. When 4-day-old chickens were infected high antibody titres persisted for 45 weeks. Chickens inoculated orally or intramuscularly with different numbers of *S. typhimurium* organisms showed graded serum IgG responses to LPS. The IgG titres in experimentally infected in-bred lines of chickens which showed greater genetic resistance to salmonella infection were significantly lower than those found in more susceptible lines. Oral and intramuscular infection with 18 different types of enterobacteria, including avian pathogenic *E. coli*, *Citrobacter* spp., *Klebsiella* spp., *Proteus* spp. and citrobacter-like organisms possessing some salmonella LPS (none possessed the O-4 antigen) and flagella antigens, did not induce *S. typhimurium* LPS-specific IgG responses. Chickens infected orally with rough or non-flagellate mutants of *S. typhimurium* did not induce high titres of LPS or flagella-specific IgG respectively. Sera obtained from *S. typhimurium*-infected chickens showed much higher titres against *S. typhimurium* LPS than with those antigens from other serotypes, including *S. enteritidis*.

INTRODUCTION

Following initial work on the value of the enzyme-linked immunosorbent assay (ELISA) in detecting *Salmonella arizonae* infection in turkeys [1, 2], a number of reports have indicated the value of different indirect ELISAs for detecting circulating antibodies to two of the most important salmonella serotypes in poultry, namely *S. typhimurium* [3–4] and *S. enteritidis* [4, 6–10].

Because high concentrations of circulating IgG persist for several months following infection with salmonellas [5, 10] the ELISA can overcome the problem in detecting infection of intermittent faecal excretion of salmonella. This has been demonstrated experimentally. In one experiment when chickens were housed with experimentally infected birds several of them displayed high IgG titres against specific antigens in the absence of detectable salmonella excretion [5]. IgM and IgA can also be detected in serum following experimental infection with *S.*

typhimurium but these antibody classes do not persist to the same extent as IgG [5, 11]. Because of its persistence ELISAs based on IgG presently have the greatest practical potential as an indication of the infection status of poultry flocks.

The ELISA based on IgG detection also has some advantages over established serological tests. It has been shown to be more sensitive than both the rapid slide agglutination test, which relies on the use of undiluted serum, and standard tube agglutination and it is at least as sensitive as the microantiglobulin test [6, 10]. Unlike the slide agglutination test, developed originally for use with *S. gallinarum* and *S. pullorum* infections, the ELISA can also be used to examine eggs, an aspect important to the poultry breeding stock, table egg and food industries [4, 9, 10, 12]. The ability of the ELISA to differentiate infections caused by salmonella serotypes has been demonstrated by using several detecting antigens prepared from different serotypes, such as lipopolysaccharide [4–6, 10] or flagella [5]. The ELISA can also be used with serum eluted from blood dried on to absorbent paper, facilitating field sampling [5, 8].

However, controversy remains over the relative merits of slide agglutination and the ELISA, suggesting that ELISAs developed by different laboratories probably vary in their sensitivity and specificity, particularly for field sampling [13–17]. Because of the great potential value of the IgG ELISA for monitoring invasive salmonella infection in poultry and to minimize such discrepancies, it is considered judicious to study each assay with experimental infections under defined laboratory conditions [17]. In this way factors affecting the performance of the assays can be assessed before each is used extensively in the field. This paper describes a further examination of an ELISA developed for use with *S. typhimurium* [5] under such conditions.

MATERIALS AND METHODS

Animals

Unless otherwise indicated salmonella-free Light Sussex chickens from a flock maintained at this laboratory were used. They were outbred and of moderate sensitivity to *S. typhimurium* [18]. In one experiment different salmonella-free, inbred lines of White Leghorn were used. These show considerable differences in susceptibility to *S. typhimurium* infection [19]. All chickens were housed and reared in conditions which have been described previously [20].

Bacterial strains

For most of the studies *S. typhimurium* F98 (phage type 14) was used because its virulence and colonization properties have been well documented [18, 20, 21]; it was resistant to nalidixic acid (Nal^r) which facilitated its enumeration in faeces. A rough (somatic antigen minus) mutant and a non-flagellate mutant of F98 Nal^r both prepared by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis by the method of Barrow and co-workers [21] were also used.

Lipopolysaccharide (LPS) was produced from smooth salmonella strains which produced no auto-agglutination in phosphate-buffered saline (PBS) and which did not agglutinate with acriflavine (0·2%). The serotypes involved were *S.*

typhimurium (group B; 1, 4, 5, 12), *S. virchow* (group C_2; 6, 7), *S. hadar* (group C_2; 6, 8), *S. eimsbüttel* (group C_4; 6, 7, 14), *S. enteritidis* (group D; 1, 9, 12) and *S. ealing* (group O 35; 35).

A number of other members of the Enterobacteriaceae which had been identified as being non-salmonella by biochemical tests (API-systems, Montalieu-Vercieu, France) were used to infect chickens. These are described in more detail below.

Unless otherwise indicated broth cultures were made in 10 ml nutrient broth (Oxoid, CM67) incubated for 24 h at 37 °C in a shaking water bath (100 strokes/min). They contained approximately 10^9 c.f.u./ml. Chickens were either inoculated orally with 0·1 ml (up to 1 week old), 0·3 ml if older, or intramuscularly with 0·1 ml of diluted or undiluted cultures.

Antigen preparation and ELISA procedure

LPS antigen was prepared by hot phenol extraction as described previously [5].

Flagella antigen was prepared by a modification of Lawn's method [22]. This involved centrifuging 1 l of a shaken broth culture at 4000 g for 10 min and resuspending the pellet in 10 ml PBS. The flagella were removed by mixing for 1 min in a Waring blender (MSE instruments) and the suspension was centrifuged at 1500 g for 30 min to remove bacterial cells. The pellet was washed in PBS and after recentrifugation the two supernatants were combined and centrifuged at 50000 g for 1 h. The pellet was resuspended in PBS and recentrifuged at 5000 g and the supernatant was retained and centrifuged at the higher speed. After resuspending the pellet in 1 ml PBS, the protein concentration was estimated colorimetrically using the Pierce Coomassie reagent (Sigma). Purity was checked by polyacrylamide gel electrophoresis with Coomassie brilliant blue staining using standard methods [23].

The ELISA method was exactly as described by Hassan and colleagues [5].

Bacterial analysis

In one experiment viable counts of inoculated salmonella were made from spleen samples. Spleens were removed aseptically from chickens killed by cervical dislocation. The organs were homogenized, diluted and plated on brilliant green agar (Oxoid, CM263) containing 25 μg/ml sodium nalidixate and 1 μg/ml novobiocin. Cloacal swabs were taken and treated as described by Smith and Tucker [20].

Experimental design

Experiment 1. Two groups of 30 chickens were housed separately. One group was maintained salmonella-free until they came into lay at 20 weeks of age. Seven hens were then bled weekly for a further 4 weeks and their eggs sampled by diluting an aliquot of each yolk 1:10 in PBS. Sera and yolk dilutions were stored at −20 °C until required. The chickens were infected orally with *S. typhimurium* F98 Nal^r at 24 weeks of age and were bled and their eggs sampled at intervals until they were 45 weeks of age. They had ceased to lay by 41 weeks.

The second group was infected orally with strains F98 Nal^r at 4 days of age. Seven chickens were bled before infection and at intervals for 45 weeks. Their sera

and yolk samples (when the hens amongst these seven came into lay) were sampled and stored as above.

Experiment 2. Eight groups of 11 chickens were housed separately. They were infected when 2 weeks of age, four groups orally with 10^8, 10^6, 10^4 and 10^2 organisms respectively of strain F98 Nal^r in 0·3 ml (diluted in nutrient broth) and four intra-muscularly with 10^7, 10^5, 10^3 and 10^1 organisms respectively of F98 Nal^r in 0·1 ml. Five chickens were bled immediately prior to infection and at intervals thereafter for 12 weeks. Three birds were killed at 4 and 7 days after infection and the viable salmonella counts in their spleen estimated. Cloacal swabs from each chicken were taken at weekly intervals for 5 weeks.

Experiment 3. Five groups each containing seven birds of different inbred lines of White Leghorn chickens designated C, 15I, 0, 6_1 and W were infected orally with *S. typhimurium* F98 Nal^r when 2 weeks old to determine what effect infection of chickens showing different susceptibilities to *S. typhimurium* infection [19] would have on antibody titres. They were bled at 4 and 6 weeks post-infection (p.i.).

Experiment 4. Five groups of 1-week-old chickens were inoculated intra-muscularly with 0·1 ml volumes of pools of cultures of different organisms from the Enterobacteriaceae. Group 1 was inoculated with 10^6 organisms each of avian pathogens *Escherichia coli* O1.K1, O2.K1, O78.K80, O18.K1H17 and an untypable strain. Groups 2–5 were all inoculated with pools of the same cultures containing 10^7 organisms. Group 2 was inoculated with two avian *Klebsiella pneumoniae* strains. Group 3 was inoculated with three uncharacterized *Proteus* strains. Group 4 was inoculated with two uncharacterized avian *Citrobacter* strains and a *C. freundii* expressing the Vi antigen. Group five was infected with a *C. freundii* strain which reached weakly with salmonella polyvalent O and polyvalent H antisera, two salmonella-like citrobacter organisms in one case reacting with poly O, poly H and Vi antigen and in the other reacting weakly with poly O antiserum. None of these organisms, all of which were isolated from chickens, agglutinated with specific O 4 or iH antiserum. A sixth group was inoculated orally with 0·3 ml of a pooled culture containing all of the above organisms from groups 1–5, each one representing approximately 5×10^6 c.f.u. in the inoculum. A seventh group, housed separately, was inoculated orally with *S. typhimurium* F98 Nal^r. Chickens were bled for 5 weeks after infection.

Experiment 5. A high-titre antiserum from *S. typhimurium* infected chickens was tested in an ELISA against LPS from different salmonella serotypes (see above) all coated on ELISA plates at 500 μg/ml.

Experiment 6. Three groups of 4-day-old chickens were infected orally with either *S. typhimurium* F98 Nal^r or with a somatic antigen-minus (rough) or non-flagellate mutant. Chickens were bled regularly and the sera examined in both LPS and flagella ELISAs

RESULTS

Experiment 1

The *S. typhimurium* LPS-specific IgG response in the serum and egg yolk of chickens which were infected with *S. typhimurium* after they had come into lay is shown in Fig. 1. The chickens were first bled at 4 days of age and then at 19–20

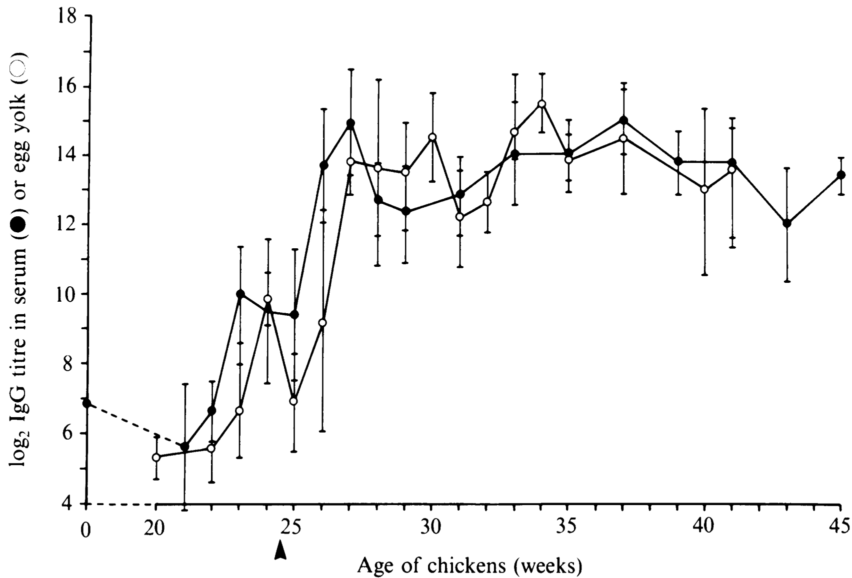

Fig. 1. LPS-specific IgG concentrations (geometric mean of seven values ± S.D.) in serum (●) and egg yolk (○) of hens infected orally with *S. typhimurium* F98 Nal^r soon after they came into lay. The arrow indicates the time of infection.

weeks, 1 week after the first egg was produced. The mean IgG titre in those sera and in the yolks of the first eggs was low ($< 1:100$, $\log_2 < 5{\cdot}6$). Before they were infected at 24 weeks the specific IgG titres increased significantly ($P < 0{\cdot}01$ for values at 23 and 24 weeks compared with 20–22 weeks), possibly as a consequence of handling. The titres then increased rapidly after infection. The standard deviation of the mean IgG titre, particularly in yolk, was very high at this time, suggesting considerable variation in the rapidity of response to infection. The mean IgG titres of serum and yolk thereafter remained high (12800–51200, $\log_2$ 13·6–15·6) during the period of lay (21 weeks' duration). The mean serum titre was still high (approximately 12800, $\log_2$ 13·5) at 45 weeks of age when the experiment was terminated. Cloacal swabs were taken from the chickens immediately prior to infection and no salmonella organisms were isolated after enrichment in selenite broth.

In the second group of chickens infected at 4 days of age mean LPS-specific IgG titres also increased rapidly soon after infection and remained high (12800–102400; $\log_2$ 13·6–16·6) for most of the 45 weeks of the experiment (data not presented). During the period of lay (21–45 weeks) serum and egg yolk IgG titres were again similar to each other. At the end of the experiment the mean serum IgG titre was approximately 25600 ($\log_2$ 14).

Experiment 2

The result of isolation of salmonella from the spleen and cloaca of chickens inoculated orally or intramuscularly when 2 weeks old with different numbers of *S. typhimurium* F98 is shown in Table 1. *S. typhimurium* organisms were isolated from the cloacal swabs of chickens inoculated with 10^8, 10^6 and 10^4 but not 10^2 organisms. Although the sample size was small the frequency of isolation reflected

Table 1. *The isolation of salmonella from the spleen and cloacal swabs from chickens inoculated orally or intramuscularly with different numbers of* S. typhimurium *F98 organisms*

Isolation of *S. typhimurium* F98 from chickens which had been inoculated						
Orally				Intramuscularly		
	Isolation of F98 from				Isolation of F98 from spleen after	
Numbers inoculated	Spleen after 4 days	Spleen after 7 days	Cloacal swabs after 1–5 weeks	Numbers inoculated	4 days	7 days
10^8	3·14[a]	3·56	6/7 (11)[b]	10^7	4·84	3·38
10^6	< 2·0[c]	3·57	4/7 (8)	10^5	4·92	3·42
10^4	< 2·0[c]	< 0·9[c]	2/7 (4)	10^3	3·04[e]	3·42
10^2	< 2·0[c]	< 0·85[c]	0/7 (0)	10^1	< 2·0[d]	3·15[e]

[a] Log_{10} median viable count of three chickens.
[b] Number of chickens yielding salmonella from cloacal swab/number of chickens swabbed. Total number of occasions salmonella isolated from swabs in parentheses.
[c] All three chickens had no detectable salmonella organisms in the spleen samples.
[d] Two of the three chickens had no detectable salmonella organisms in the spleen samples.
[e] One of the three chickens had no detectable salmonella organisms in the spleen samples.

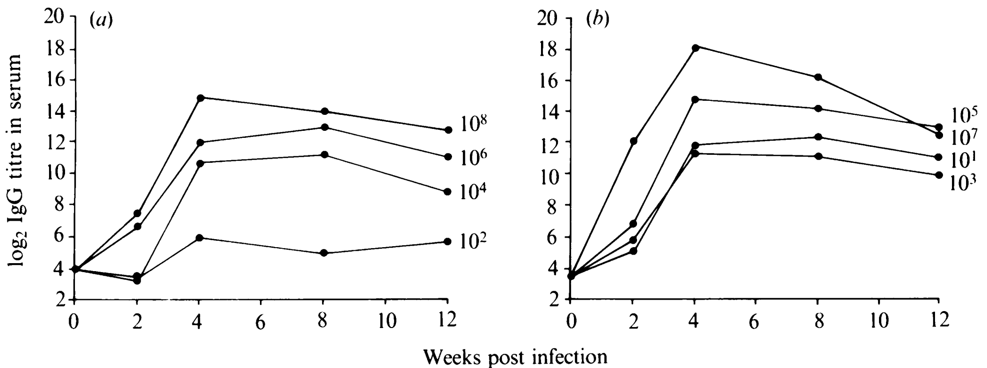

Fig. 2. LPS-specific IgG concentrations (geometric mean of five values) in serum of chickens infected with different inoculum sizes of *S. typhimurium* F98 Nal^r when 2 weeks old. (*a*) Chickens infected orally; (*b*) chickens infected intramuscularly. The numbers of salmonella organisms inoculated in each case is shown at the right of each figure.

the inoculum size. The number of organisms isolated from the spleens increased between 4 and 7 days after infection but none was detected in the spleens of chickens inoculated with 10^4 or 10^2 organisms, however no enrichment was carried out. The LPS-specific IgG responses in the serum of these chickens is shown in Fig. 2. Large increases in IgG titres were seen at 4 weeks p.i. in the group inoculated with 10^4–10^8 organisms. These increases were statistically significantly higher ($P < 0{\cdot}01$) than the small increases seen in the chickens inoculated with 10^2 organisms. From a combination of the bacteriological and serological data it

Table 2. *Antibody titres obtained in LPS ELISA with sera from inbred chicken lines infected with* S. typhimurium *F98 Nal*[r]

Chicken line	$\log_2$ geometric mean IgG titre of seven birds ± S.D.[a]	LD_{50} value[b] ± S.D.
C	16·2 ± 2·3	0·84 ± 0·23
15I	16·2 ± 1·78	< 1·0
O	13·8 ± 0·84	N.D.
6_1	11·6 ± 1·22	4·53 ± 0·20
W	9·6 ± 3·54	2·28 ± 0·43

[a] Chickens inoculated orally with 3×10^8 c.f.u. in 0·3 ml of *S. typhimurium* F98 Nal[r] at 2 weeks of age and bled 4 weeks later.

[b] Groups of five newly hatched (less than 24 h old) chickens inoculated intramuscularly with 0·1 ml volumes of decimal dilutions of broth culture of *S. typhimurium* F98. LD_{50} calculated as described previously [19].

[c] N.D., not done.

appeared that although these latter chickens had been inoculated with 10^2 organisms they had not become infected.

Although at 4 days after infection these were some gradation in the numbers of organisms isolated from the spleen of chickens inoculated intramuscularly with different inoculum sizes the difference had almost disappeared by 7 days p.i. Statistically significant increases in specific IgG titres ($P = < 0{\cdot}01$) were seen in all groups at 4 and 8 weeks after infection (increases above those values seen in the chickens inoculated orally with 10^2 organisms), the highest titres being seen in the chickens inoculated with the highest number of organisms. The IgG titres had decreased slightly by 12 weeks p.i. and there was less difference in the mean titres of the four groups.

Experiment 3

The $\log_2$ geometric mean LPS-specific IgG titres obtained from different lines of chickens inoculated orally with *S. typhimurium* F98 and bled 4 weeks later are shown in Table 2. The titres obtained when the birds were re-bled 2 weeks later were very similar. There was some evidence that the lowest titres were seen in those lines which were resistant to systemic *S. typhimurium* infection and *vice versa*. The IgG titres obtained from W and 6_1 lines were significantly lower ($P < 0{\cdot}01$) than those obtained from the more susceptible lines C and 15I.

Experiment 4

In all six groups of chickens inoculated intra-muscularly or orally with 18 different members of the Enterobacteriaceae, a few of which gave weak agglutination reactions with commercial polyvalent salmonella antisera, very low titres of antibody were obtained (12·5–50, $\log_2$ 3·6–5·6). In contrast a mean titre of $\log_2$ 12·8 was obtained with sera from chickens inoculated with *S. typhimurium*.

Experiment 5

The IgG titre obtained with a high titre serum from a chicken from experiment 1 using LPS from *S. typhimurium* was 51200. The titres obtained with the same serum but using, as coating antigen, LPS from smooth strains of *S. enteritidis*

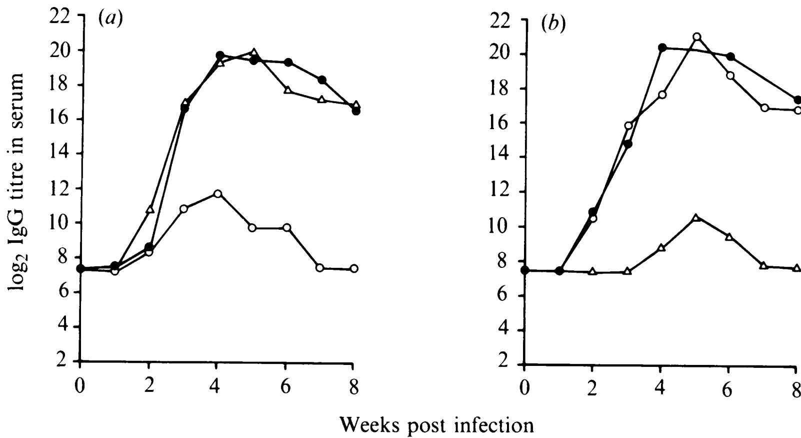

Fig. 3. (*a*) LPS-specific and (*b*) flagella-specific IgG concentrations in serum of chickens infected at 4 days of age with *S. typhimurium* F98 Nal^r or with a rough mutant or non-flagellate mutant of this strain. Serum obtained from five chickens at each point. Chickens inoculated with parent strain (●), rough strain (○), non-flagellate strain (△).

(O 1, 9, 12), *S. virchow* (O 6, 7), *S. eimsbüttel* (O 6, 7), *S. hadar* (O 6, 8) and *S. ealing* (O 35) were 800, 800, 1600, 400 and 400 respectively.

Experiment 6

The IgG titres obtained with serum from chickens infected with either the parent strain or a rough- or non-flagellate-mutant of *S. typhimurium* F98 Nal^r and tested against LPS and flagella preparations are shown in Fig. 3. When LPS was used as a coating antigen much lower titres were obtained with sera from chickens infected with the rough strain than with sera from chickens infected with either the parent or with the non-flagellate strain. Similarly, with flagella as the coating antigen lower titres were obtained with sera from chickens infected with the non-flagellate strain compared with sera from chickens infected with the parent or with the rough strain.

DISCUSSION

This paper reports the results of examining further the potential for an indirect ELISA based on IgG as a means of serological monitoring of poultry flocks for *S. typhimurium*. Some of the factors which might produce a variable performance when using samples obtained from the field have been examined. While in this work only infections caused by *S. typhimurium* were used, it is likely that many of the results are relevant to other salmonella serotypes that are invasive for poultry including *S. enteritidis* and *S. gallinarum–S. pullorum* [24].

As found previously, *S. enteritidis* IgG titres persist for many months [9, 10]. Previous studies with *S. typhimurium* indicated that circulating IgG was no indication of the bacteriological status of chickens. The disadvantage of persistent high IgG titres is that chickens which are not infectious may be regarded as so because of their serological status. However, since some recent work [25] has

indicated that hens can lay *S. enteritidis* infected eggs well after faecal excretion has ceased, persistent high IgG levels may not be a major problem since environmental contamination may still exist in the absence of active excretion, indicating a potential source of reinfection. Serum and egg yolk titres were very similar as found previously with *S. enteritidis* [9] reinforcing the value of assaying eggs in breeder and laying flocks as an alternative to serum [4]. The only discrepancy occurred as IgG concentrations increased soon after infection when the titres in yolk rose a little later than those in serum. The increases in titre seen immediately prior to infection may occur as a result of stress arising from handling. The effects of stress should be examined in greater detail as an additional factor which may affect antibody response. In a separate study with *S. gallinarum* [24] 'coming into lay' also appeared to produce a non-specific increase in antibody titres.

The graded serological responses obtained with different doses of *S. typhimurium* indicated that variations in exposure which must occur in the field could, together with differences in time between exposure to infection and sampling, be one of the factors accounting for variation in serum IgG concentrations. The exception to this is infection in very young chicks where even small inocula result in considerable antigen exposure as a result of the massive microbial multiplication that is able to occur in the alimentary tract soon after hatching. The graded response also indicates the need to evaluate the test as a flock test since, in the absence of salmonella excretion, intermediate IgG concentrations present in individual birds would be very difficult to interpret.

The genetic susceptibility of a line of chickens may also affect the serological response although with an organism as invasive as *S. enteritidis* phage type 4 [6, 9, 24] this may not be a problem since enough bacteria should enter the tissues to stimulate a good antibody response. If lower titres are generally found in more resistant lines this could increase the numbers of samples required from a flock to determine the extent of infection. Some recent unpublished findings (McLeod, personal communication; Nicholas, personal communication) indicate that the number of samples required to find serological evidence of *S. enteritidis* in some flocks is small and is well below the hundreds of swab samples required to detect the low levels of bacterial excretion normally encountered.

As found previously [5] the ELISA is highly specific. No cross reactions were found with serum collected following oral or parenteral inoculation with a wide range of antigenically related enterobacteria. Some of these would presumably pose little problem in flocks because they are non-invasive and would not generate high concentrations of circulating antibody. The specificity of the response using both LPS and flagella as antigens was also shown by the absence of any response to LPS and flagella in mutants not producing them. Rough strains of salmonella are occasionally isolated in the field and these may conceivably produce false negative results when LPS is used as antigen. A reasonably strong antibody response to flagella however, would be expected.

The IgG ELISA for *S. typhimurium* has been shown to be sensitive and specific enough for extensive evaluation in the field. There is also no reason to believe that, for most of the experiments reported on here, other invasive serotypes, including *S. enteritidis*, would behave in a markedly different way.

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