

Serological response of chickens to *Salmonella enteritidis* infection

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SUMMARY

Fifty-eight sera, from 29 chickens originating from two layer flocks known to be naturally infected with *Salmonella enteritidis* phage type (PT) 4, were examined for antibodies to *S. enteritidis*. Using the techniques of immunoblotting and ELISA, antibodies to the lipopolysaccharide (LPS) of *S. enteritidis* were detected in 43 of 58 sera. Antibodies were of the IgG class and bound to the *S. enteritidis* LPS antigen O = 12. Bacterial agglutination reactions using whole-cell preparations of *S. enteritidis* and *S. pullorum*, correlated with anti-LPS antibody reactions as detected by immunoblotting and ELISA. A rapid means of screening chicken sera for antibodies to the LPS of *S. enteritidis* as an indicator of infection is discussed.

INTRODUCTION

In 1988 *Salmonella enteritidis* was the most common serotype isolated from humans in England and Wales (1). Subdividing strains of *S. enteritidis* by phage typing (2) showed that one particular phage type (PT), PT 4, was the most frequently isolated. Chickens are the main reservoir and vehicles of infection are poultry meat and shell eggs (3–5), particularly when used in recipes incorporating uncooked eggs (6).

Some chicken flocks have been shown to be infected with *S. enteritidis* PT4 and may lay infected eggs (7, 8); however, the exact proportion of the 50 million commercial laying hens infected with *S. enteritidis* is unknown. The problems associated with assessing the levels of infection of laying flocks with *S. enteritidis* and possible action that might be taken to prevent contaminated eggs entering the human food chain was addressed during a House of Commons Agricultural Committee (9). Identification of infected chicken flocks was considered essential, and serological and bacteriological approaches were advocated.

Isolating *S. enteritidis* PT4 from chicken tissues and eggs would provide a definitive means of identifying infected flocks; however, this approach might prove economically prohibitive. In contrast, an immunological test, although not as definitive as a bacteriological examination, would provide evidence of infection

with *S. enteritidis* without destructive sampling of chicken flocks. In the present study sera from two small flocks of chickens naturally infected by *S. enteritidis* PT4 were used to assess the antibody response of naturally infected birds to *S. enteritidis* antigens and to investigate the possibility of applying a serological test for the screening of chicken flocks.

MATERIALS AND METHODS

Birds

The 29 birds were housed individually in wire mesh-floored cages and were fed pelleted commercial ration and allowed to drink *ad libitum*. Artificial light was provided on a 12 h on, 12 h off cycle.

Bacteria

Eight strains belonging to the genus *Salmonella* (Table 1.) and one strain of *Escherichia coli* (E32511) were used in this study. *S. enteritidis* strain P132344 was isolated from chicken pericardial fluid (4). Strains were stored on Dorset egg slopes at room temperature in the Division of Enteric Pathogens. Bacteria were grown in Trypticase Soy Broth (BBL) for 16 h (37 °C) with shaking (120 rpm).

Blood samples

Fifty-eight sera were obtained from 29 birds comprising two flocks of egg-laying hens, designated P and Y. Fifteen birds from flock P and 14 birds from flock Y were initially bled on 22 February 1989 and again on 12 and 14 April respectively. Sera were separated and stored at -10 °C.

Whole-blood samples were also taken from flock P using heparin as anticoagulant.

Bacterial examination of birds and eggs

Following blood sampling of flock Y, 11 of the 14 birds were killed by inhalation of carbon dioxide. Necropsy was carried out immediately and cloacal swabs and samples of liver, spleen, jejunum, oviduct, ovule and ovary tissues were taken for bacteriological examination. Eggs layed by birds in both flocks were also cultured for *S. enteritidis* PT4.

Histological examination

The following organs were fixed in 10% buffered neutral formalin: liver, kidneys, crop, ileum, caecum, colon, oviduct and brain. Tissues were processed by standard methods and embedded in paraffin wax. Section were cut at 5 µm and stained with haemotoxylin and eosin.

Outer membranes

Outer membranes (OMs) were prepared from sonicated (150 W, 3 min, 0°C) bacteria as described previously (10) by selectively solubilizing the cytoplasmic membrane with Sarkosyl (BDH, Poole, Dorset). For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 30 µg of OM protein (OMP) was applied per lane.

Lipopolysaccharide

Lipopolysaccharide (LPS) was isolated from *S. enteritidis* strain P132344 using hot-phenol (11). Purity of the resultant LPS was assessed by staining SDS-PAGE profiles with silver for carbohydrate (12) and for contaminating proteins (13). For ELISA tests a batch of *S. enteritidis* LPS preparation was made and replicate aliquots containing 10 µg LPS were stored at -10 °C.

Flagella

Bacteria were grown as outlined above. Flagella were prepared by incubating bacteria in phosphate buffered saline (PBS) at 60 °C for 30 min, followed by sedimentation of bacterial cells (5000 g, 30 min, 4 °C) and then flagella (40000 g, 30 min, 4 °C). The protein concentration of bacterial preparations was determined by the method of Lowry (14).

SDS-PAGE immunoblotting

Bacterial proteins and LPS were separated by SDS-PAGE (15) as described previously (16), using a constant current (50 mA) for 3.25 h. Profiles were stained with either Coomassie blue (17) or silver (12, 13) or used for immunoblotting.

Immunoblotting

OM, LPS and flagella profiles were transferred onto nitrocellulose sheets and reacted with sera (30 µl per lane) as described previously (16). For the detection of IgM antibodies, profiles were reacted with a goat anti-chicken IgM antiserum (Nordic Immunology Ltd) followed by a ¹²⁵Iodine radiolabelled donkey anti-goat Ig antibody (Sigma Chemical Co.). Antibodies of the IgG class were detected using a radiolabelled goat anti-chicken IgG antibody (Southern Biotechnology). The binding of radiolabelled antibodies was detected by autoradiography (16).

ELISA

Enzyme-linked immunosorbent assays were performed as described (16). ELISA plates were coated with 0.1 µg of LPS preparation in 100 µl coating buffer per well. Sera were diluted (× 500) in PBS. IgM antibodies were detected using a goat anti-chicken IgM antiserum (Nordic Immunology Ltd) followed by an alkaline phosphatase-conjugated anti-goat antibody (Sigma Chemical Co). IgG antibodies were detected with an alkaline phosphatase-conjugated goat anti-chicken IgG antibody (Southern Biotechnology). Antibodies bound to coated LPS were detected and quantified by adding the enzyme substrate (*p*-nitrophenol phosphate, Sigma Chemical Co) and reading the resultant colour absorbance at 405 nm (A_{405}).

Salmonella whole-cell antigens and slide agglutinations

A preparation of *S. pullorum* stained antigen 'pullorum antigen' was obtained from the Ministry of Agriculture Fisheries and Food, Weybridge, England. A *S. enteritidis* stained antigen 'enteritidis antigen' was prepared in this laboratory. Using strain P132344, bacteria from 1 litre of broth culture were sedimented (5000 g, 30 min, 4 °C), washed three times in PBS and incubated in PBS containing 1%

(v/v) formalin (4 °C, overnight). Cells were sedimented as above and stained with 1% crystal violet in PBS (1 h, 20 °C), and the absorbance at 621 nm (A_{621}) adjusted to the same as the purchased pullorum antigen.

For slide agglutination experiments 0.1 ml of serum was mixed with 0.1 ml of pullorum or enteritidis antigen preparations. Any serum giving an agglutination reaction within 2 min of mixing was considered antibody positive. An antigen prepared from *E. coli* strain E32511 (O 157.H7) was used as a negative control.

RESULTS

Bacteriology

Bacteriological examination of the 11 birds from flock Y demonstrated *S. enteritidis* PT4 in the liver and spleen of bird 44, in the jejunum of bird 48, and in the spleen, liver, cloaca, oviduct, ovules and ovary of bird 49; this organism was also detected in eggs layed by bird 48. Eggs laid by birds 2, 5, 7, 9, 11, 12 and 14 from flock P were also found to be infected by *S. enteritidis* indicating that at least 10 of the 29 birds examined were infected with this organism.

Histopathology

The lamina propria of the ileum, caecum and colon had a variable infiltrate of lymphoid cells but the epithelium was intact at all sites examined. This was similar in extent to the pattern seen in the intestine of normal chicks taken as controls. There was a low level of infestation of tapeworms and round worms. Occasional lymphoid foci were present in the liver and kidneys of four infected birds.

Antibodies to S. enteritidis OMs

Sera were initially reacted with OM SDS-PAGE profiles prepared from *S. enteritidis* PT4 strain P132344 (Fig. 1, lane 1) by immunoblotting. Using an anti-chicken IgG immunoglobulin preparation, 43 of the 58 sera were found to contain antibodies of the IgG class reacting with what appeared to be long-chain LPS (Fig. 1, lane 2). The remaining 15 sera were found to react with a 40 kDa OMP (Fig. 1, lane 3) arrowed in lane 1. Immunoblot reactions with purified LPS (Fig. 1, lane 4) showed that the predominant antibody response was to LPS (Fig. 1, lane 5). An IgM response was not detected to either OMs or purified LPS in any of the sera tested. Selected strains of *Salmonella* spp. listed in Table 1 were used to identify the main antigenic site(s) on *S. enteritidis* LPS. Sera from chickens giving a positive immunoblot reaction with *S. enteritidis* LPS (antigenic formula 1, 9, 12) were reacted by immunoblot with LPS isolated from *S. typhimurium* (1, 4, 5, 12), *S. dublin* (1, 9, 12), *S. plymouth* (9, 46), *S. gallinarum* (1, 9, 12) and *S. pullorum* (1, 9, 12). The only LPS type not recognised by test sera was that of *S. plymouth*.

LPS ELISA

Qualitative serum antibody reactions to LPS detected by immunoblotting were quantified by an ELISA using a purified LPS preparation (0.1 µg per well). Sera from birds from flock P gave a mean ELISA value of 0.38 (± 0.20) and 0.35

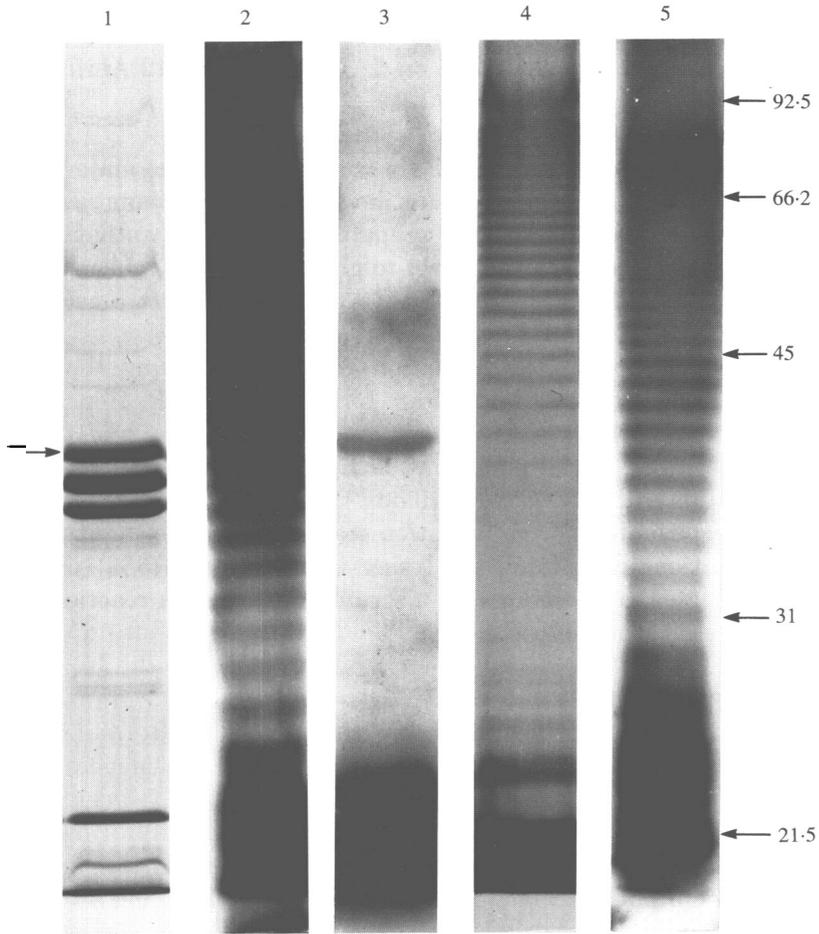


Fig. 1. When chicken sera were reacted with OM profiles of *S. enteritidis* strain P132344 (e.g. lane 1) 43 of 58 sera reacted with LPS (lane 2) whilst the remaining 14 sera reacted with an OMP of 40 kDa (eg. lane 3) arrowed in lane 1. Reacting sera with purified LPS (lane 4) showed that antibodies were indeed reacting with LPS (lane 5). 30 μ g protein used run per lane, 5 μ g of LPS used per lane and 30 μ l of serum used for immunoblotting. The values given at the side of the figure are in kilodaltons.

Table 1. *Strains of Salmonellae used*

| Strain no. | Organism | Antigenic properties |
|------------|---------------------------|----------------------|
| P132344 | <i>S. enteritidis</i> PT4 | 1, 9, 12: g, m |
| S108797 | <i>S. typhimurium</i> | 1, 4, 5, 12: i: 1, 2 |
| S108881 | <i>S. dublin</i> | 9, 12: g, m: - |
| S107861 | <i>S. plymouth</i> | 9, 46: d: z6 |
| S075566 | <i>S. gallinarum</i> | 9, 12: -: - |
| P136267 | <i>S. pullorum</i> | 9, 12: -: - |
| JT3192 | <i>S. pontypriidd</i> | 18: g, m |
| JT689 | <i>S. godesburg</i> | 30: g, m |

(± 0.25) for blood samples taken on 22 February and 14 April respectively. Similarly, Sera from birds from flock Y gave ELISA values of 0.44 (± 0.27) and 0.48 (± 0.26) for blood samples taken on 22 February and 12 April respectively.

Salmonella agglutinations

By immunoblotting and ELISA LPS was shown to be the major *S. enteritidis* antigen recognized by chicken serum antibodies. Using a commercial 'pullorum' antigen preparation and a laboratory prepared 'enteritidis' antigen 18 sera from flock P and 14 from flock Y were shown to give a positive agglutination reaction within the designated 2 min. None of the 58 sera reacted with a negative control antigen prepared from *E. coli* strain E32511.

We also investigated an agglutination reaction involving the 'enteritidis' antigen and samples of whole blood from birds from flock P. Heparinized blood samples from birds with high titre serum antibodies to *S. enteritidis* agglutinated both the 'enteritidis' and 'pullorum' antigen preparations. However blood samples from birds with low serum antibody titres were not readily detected using whole-blood agglutinations. Agglutination reactions were repeated using the two antigen preparations containing the lysing agent saponin (5%). Following the lysis of erythrocytes most sera giving a serum agglutination reaction also gave a reaction with lysed whole-blood.

Antibodies to flagellar antigens

A strain of *S. pontypriidd* and *S. godesburg* were used to detect possible antibodies to *S. enteritidis* flagella. All three strains have the flagellar (H) antigens g, m but only *S. enteritidis* has the LPS O antigens 1, 9, 12. Flagella were isolated from *S. pontypriidd* and *S. godesburg* and used for immunoblotting and ELISA tests. Antibodies to flagella were not detected by immunoblotting and significant ELISA titres were not detected. When ELISA titres from the 43 sera giving a positive LPS immunoblot reaction were compared to the remaining 15 immunoblot-negative sera using student's *t* test, no significant difference was detected ($P > 0.1$).

Analysis of results

Using all 58 sera patterns of serum antibody responses detected by immunoblotting ELISA and agglutination were assessed. The results in Fig. 2 show 49 of the 58 sera listed in order of decreasing serum antibody titre. Comparing immunoblotting data with ELISA results showed that all sera giving positive immunoblot reactions (dotted bars in Fig. 2) had ELISA readings > 0.2 except for one serum with an ELISA value of 0.14. When agglutination reactions were included in Fig. 2, with the exception of two sera a direct correlation was detected between a qualitative agglutination reaction (filled bars in Fig. 2) high ELISA titres and positive immunoblot reactions. The two sera giving high ELISA values but not agglutinating either antigen preparations were from the same bird (Y36). The serological data for chickens infected with *S. enteritidis* and/or laying infected eggs are shown in Table 2. Nine of the ten infected birds contained antibody titres > 0.2 (A_{405}) and gave antibody-positive reactions with LPS by both immunoblotting and agglutination techniques.

Table 2. Serology of chickens containing *S. enteritidis* in tissues* and/or laying infected eggs

| Tissues from | ELISA titre† | Immunoblot reaction† | Agglutination reaction† |
|--------------|--------------|----------------------|-------------------------|
| Y44 | 0.69, 0.57 | +, + | +, + |
| Y48 | 0.74, 0.44 | +, + | +, + |
| Y49 | 0.98, 0.87 | +, + | +, + |
| Eggs from | | | |
| Y48 | 0.74, 0.44 | +, + | +, + |
| P2 | 0.43, 0.26 | +, + | +, + |
| P5 | 0.00, 0.03 | -, - | -, - |
| P7 | 0.41, 0.54 | +, + | +, + |
| P9 | 0.54, 0.57 | +, + | +, + |
| P12 | 0.40, 0.39 | +, + | +, + |
| P14 | 0.76, 0.60 | +, + | +, + |

* See results section for details of tissues infected.

† Primary, secondary serum samples, see Materials and Methods.

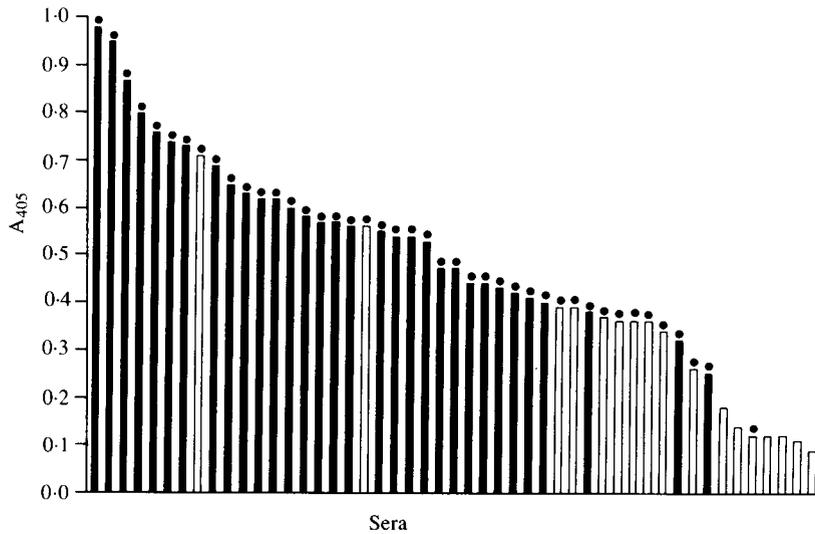


Fig. 2. Histogram showing 49 of the 54 sera listed in descending ELISA titre. Antibody reaction with LPS by immunoblotting (●) were not generally detected in sera giving an ELISA titre < 0.2 (A_{405}). Of the 43 sera showing an antibody reaction by immunoblotting (●) 32 sera gave a positive agglutination reaction (filled bars).

DISCUSSION

This present study examined the serum antibody response of chickens naturally infected with *S. enteritidis*. Three of the 11 birds autopsied were infected with *S. enteritidis* PT4 and 7 of the chickens laid eggs with the organism in the egg contents. Immunoblotting showed that some birds had serum antibodies of the IgG class to the LPS of *S. enteritidis* which enabled the development of an ELISA using purified LPS. Although a range of ELISA titres was detected only one serum

with an ELISA titre of < 0.2 (A_{405}) gave a reaction by immunoblotting. Sera which did not react with LPS were found to contain antibodies to a 40 kDa OMP. This protein was thought to be ompF since an OMP of 38.9 kDa has been demonstrated to be ompF in all *Salmonella* spp. (18). A reaction with this protein in sera containing antibodies to LPS was obscured by comigrating LPS bands. The observed serum antibody reaction with the LPS of serovars of *Salmonella* containing the antigen O = 12 but not O = 9 indicated that the predominant epitope on *S. enteritidis* and other serotypes was the α -D-glucose and D-galactose sugars of antigen O = 12 (19, 20). The reactions of serum antibodies with the LPS of both *S. enteritidis* and *S. pullorum* detected by immunoblotting and ELISA were confirmed by agglutination reactions with whole-cell preparations of *S. enteritidis* and *S. pullorum* which contain the same LPS structures (21). From our studies 9 of 10 chickens, showed to be infected with *S. enteritidis* PT4 or lay infected eggs, contained high-titre serum antibodies to the LPS of *S. enteritidis*. A serological test employing immunoblotting, ELISA and/or agglutination reactions would have correctly identified 90% of birds infected with *S. enteritidis*. The remaining eight chickens from flock Y shown to have readily detectable levels of anti-LPS antibodies might have had a transient infection with *S. enteritidis* within immunological memory or might have contained the organism at levels below detection. A serological approach has been used to detect evidence of infection of chickens by *S. typhimurium*, *S. thompson* and *S. pullorum* (22–24). Our studies suggest that a serological test would also provide evidence of infection of chickens with *S. enteritidis*. An agglutination reaction involving either the 'pullorum' or 'enteritidis' antigens could be used to detect serum antibodies to *S. enteritidis*. We further showed that agglutination reactions with the 'enteritidis' antigen could be performed using whole blood in the presence of a lysing agent such as saponin.

This study investigated the serum immune response of chickens to *S. enteritidis*. We have shown that a simple agglutination reaction using lysed whole blood correlates with high-titre serum antibodies and could be used to screen chicken sera for antibodies to serotypes of *Salmonella* possessing the O = 12 antigen. In the light of the current problems of screening chickens for evidence of infection with *S. enteritidis* serum antibody tests such as those described here could be used as a primary screening followed by bacteriological examination of birds giving a strong antibody reaction.

REFERENCES

1. Threlfall EJ, Rowe B, Ward LR. Subdivision of *Salmonella enteritidis* phage types by plasmid profile typing. *Epidemiol Infect* 1989; **102**: 459–65.
2. Ward LR, De Sa JDH, Rowe B. A phage-typing scheme for *Salmonella enteritidis*. *Epidemiol Infect* 1987; **19**: 291–4.
3. Coyle EF, Palmer SR, Ribeiro CD, et al. *Salmonella enteritidis* phage type 4 infection: Association with hens' eggs. *Lancet* 1988; ii: 1295–7.
4. Chart H, Threlfall EJ, Rowe B. Virulence of *Salmonella enteritidis* phage type 4 is related to the possession of a 38 MDa plasmid. *FEMS Microbiol Letts* 1989; **58**: 299–304.
5. Smith JWG. Memorandum of evidence to the agricultural committee inquiry on salmonella in eggs. *Public Health Laboratory Service Microbiology Digest* 1989; **6**: 1–9.
6. Anonymous. *Salmonella enteritidis* phage type 4: chicken and egg. *Lancet* 1988; ii: 720–2.
7. Hopper SA, Mawer S. *Salmonella enteritidis* in a commercial layer flock. *Vet Rec* 1988; **123**: 351.

8. Humphrey TJ, Cruickshank JG, Rowe B. *Salmonella enteritidis* phage type 4 and hens' eggs. *Lancet* 1989; **i**: 281.
9. Anonymous. *Salmonella* in eggs. Report and Proceedings of the House of Commons Agricultural Committee, 1989.
10. Chart H, Griffiths E. Antigenic homology of the ferric enterobactin receptor protein of *Escherichia coli*. *J Gen Microbiol* 1985; **131**: 1503–9.
11. Westphal O, Jahn K. Bacteriological lipopolysaccharide: extraction with phenol-water and further applications of the procedure. In: Whistler R, ed. *Methods in carbohydrate chemistry*. New York: Academic Press, 1965; 83–91.
12. Tsai C-M, Frasch CE. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. *Anal Biochem* 1982; **119**, 115–9.
13. Wray W, Boulikas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. *Anal Biochem* 1981; **18**: 197–203.
14. Lowry OH, Rosenbrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–75.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond* 1970; **227**: 680–5.
16. Chart H, Scotland SM, Rowe B. Serum antibodies to *Escherichia coli* serotype O 157. H7 in patients with hemolytic uremic syndrome. *J Clin Microbiol* 1989; **27**: 285–90.
17. Griffiths E, Stevenson P, Thorpe R, Chart H. Naturally occurring antibodies in human sera that react with the iron regulated outer membrane proteins of *Escherichia coli*. *Infect Immun* 1985; **47**: 808–13.
18. Nakae T. Outer membrane permeability of bacteria. *Crit Revs Microbiol* 1986; **13**: 1–61.
19. Hellerqvist GG, Lindberg B, Svensson S. Structural studies of the O-specific side chains of the cell wall polysaccharides from *Salmonella typhi* and *Salmonella enteritidis*. *Acta Chem Scand* 1969; **23**: 1588–96.
20. Luderitz O, Freudenberg MA, Galanos C, Lehman V, Rietschel ET, Shaw DH. Lipopolysaccharides of gram negative bacteria. *Curr Top Membr Trans* 1982; **17**: 79–151.
21. Luderitz O, Staub AM, Westphal O. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae*. *Bacteriol Revs* 1966; **30**: 192–245.
22. Thain JA, Cullen GA. Detection of *Salmonella typhimurium* infection in chickens. *Vet Rec* 1978; **102**: 143–5.
23. Williams JE, Whittmore AD. Serological response of chickens to *Salmonella thompson* and *Salmonella pullorum* infections. *J Clin Microbiol* 1979; **9**: 108–14.
24. Thain JA, Blandford TB. A long-term serological study of a flock of chickens naturally infected with *Salmonella pullorum*. *Vet Rec* 1981; **109**: 136–8.