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Development and Application of Enzyme-Linked Immunosorbent Assay for Specific Detection of *Salmonella enteritidis* Infections in Chickens Based on Antibodies to SEF14 Fimbrial Antigen

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A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies to the SEF14 fimbrial antigen (SEF14-DAS ELISA) and was evaluated for its use in the specific detection of chicken flocks infected with *Salmonella enteritidis*. The SEF14-DAS ELISA successfully discriminated between chickens experimentally infected with *S. enteritidis* and those infected with *S. panama* or *S. typhimurium*, although the SEF14 responses in adult birds infected with *S. enteritidis* were detectable but low. In contrast, ELISAs used to detect antibodies to lipopolysaccharide (LPS) and flagella were unable to discriminate between the infected groups of chicks and adult birds infected with different *Salmonella* serotypes. LPS and flagellar responses were low and variable in chicks, whereas in adult hens they were found to be consistently strong. When flocks naturally infected with *S. enteritidis* were tested by the SEF14-DAS ELISA and ELISAs to detect LPS and flagellar antibodies, it was found that they could all identify the infected flocks, although there was little correlation between individual serum samples. The study shows that the SEF14-DAS ELISA may offer advantages over existing assays with comparable sensitivities coupled with higher specificities for the serological detection of *S. enteritidis*-infected chicken flocks.

For the last 10 years, *Salmonella enteritidis* has been one of the dominant *Salmonella* serotypes isolated from humans with food poisoning in many countries including the United Kingdom and the United States (10). This phenomenon has been associated with an increase in the numbers of poultry and eggs contaminated with this serotype (9, 11).

To reduce the risk of vertical transmission in poultry, European Directive 92/117/EEC lays down harmonized rules for the control of *Salmonella* isolates in breeding flocks, including the frequencies and types of tests and the actions required.

Bacteriological techniques for the isolation of salmonellas from clinical and environmental samples are laborious, lengthy, and expensive. They may not identify all *S. enteritidis*-infected flocks because of the intermittent nature of salmonella excretion (21) and the number of samples that can be processed. However, chickens infected with invasive serotypes like *S. enteritidis* develop a persistent immunoglobulin G response to the infecting organism (2). Thus, mass serological screening for flocks infected with *S. enteritidis* offers a cheaper and more practical alternative, provided that it gives at least the same performance as bacteriological methods. This option is available in the European Directive, provided that bacteriological confirmation is carried out.

Enzyme-linked immunosorbent assay (ELISA)-based serological tests are most suitable for the mass screening of chicken flocks and have been developed for the detection of salmonella antibodies (2). Most ELISAs currently in use for the detection of *S. enteritidis*-infected flocks identify antibodies to either crude surface-extracted antigens or lipopolysaccharide (LPS), and while most reach the required sensitivity, they may give rise to false-positive results and are also unable to discriminate between *Salmonella* serotypes (1, 2, 8). ELISAs that use flagellar antigens have also been successfully developed (18, 19) and have been shown to be highly specific; a monoclonal antibody (MAb)-based blocking assay is now used in the mass screening of breeding and commercial laying flocks in The Netherlands (19). However, it is recognized that flagellar antibodies may not persist in the bird as long as LPS-specific antibodies, which may reduce the overall sensitivities of these assays (2). Outer membrane antigens derived from *S. enteritidis* have also been used in ELISAs to successfully identify experimentally infected chickens. However, their cross-reactivity with other *Salmonella* serotypes and use in the detection of naturally infected birds were not evaluated (5).

A novel fimbrial antigen termed SEF14 was first described on strains of *S. enteritidis* (7, 14). SEF14 is produced mainly by *S. enteritidis* and *S. dublin* strains and thus can be regarded as specific to *S. enteritidis* among salmonella isolates from poultry (13, 15). SEF14 fimbriae are expressed by all *S. enteritidis* strains tested so far (13), and preliminary results indicate that chickens develop antibodies to SEF14 following infection with *S. enteritidis* (12). The present study used an MAb-based ELISA for the characterization of the SEF14 antibody responses in chicks and adult birds, and the test was then evaluated for the specific serological detection of chicken flocks infected with *S. enteritidis.*

MATERIALS AND METHODS

Bacterial strains. *S. enteritidis* LA5, 1246/89, and 486/86 (phage type 4), *S. enteritidis* S11146/93 (phage type 8), *S. typhimurium* 2391N, and *S. panama* S10173/93 were obtained from the reference collection at the Central Veterinary Laboratory, Addlestone, United Kingdom. All of the strains were stored on Dorset egg slopes.

Preparation of the SEF14 fimbrial antigen. *S. enteritidis* 486/86 was chosen for the bulk production of SEF14 fimbriae because of its continuous high level of expression of SEF14 when it is grown on Sensitest agar (Oxoid, Basingstoke, United Kingdom). Organisms were grown on Sensitest agar for 18 h at 37°C and were harvested into phosphate-buffered saline (PBS; pH 7.2). SEF14 was then removed from the bacterial surface by heating the suspension at 60°C for 30 min; this was followed by mild sonication in a water bath for a further 5 min. The cells were sedimented by centrifugation at 3,000 × *g*, and the supernatant was precipitated with dry ammonium sulfate at 25% (wt/vol) and 60% (wt/vol) saturation. The precipitated proteins were dissolved in a small volume of distilled water and were stored at −20°C (crude SEF14). Further purification was carried out on

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the crude SEF14 by anion-exchange chromatography with a DEAE-Sepharose CL 6B (Pharmacia LKB, Uppsala, Sweden) column (25 by 100 mm) which was equilibrated with 0.02 M phosphate buffer (pH 7.5). The sample was applied to the column at a flow rate of 1 ml/min, and the wash and elution steps were performed at 5 ml/min. Five-milliliter fractions were eluted with equilibration buffer containing 1 M sodium chloride and were collected throughout the run. Finally, fractions containing SEF14 were applied to a high-pressure size-exclusion GF-450 Zorbax (Anachem, Luton, United Kingdom) column (25 by 250 mm). The buffer used was 0.2 M phosphate (pH 7.0) with a flow rate of 1 ml/min. One-milliliter fractions were collected over a period of 20 min (pure SEF14) and were stored at −20°C.

The purities of the SEF14 preparations were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using 12.5% gels under reducing conditions (6).

Preparation of LPS and flagellar antigens. A lyophilized preparation of LPS derived from *S. enteritidis* (Sigma Chemical) was used in ELISAs for the determination of LPS antibodies in chicken sera. *S. enteritidis* flagellar antigen was prepared as described previously (4). In brief, *S. enteritidis* LA5 was grown in nutrient broth for 18 h at 37°C, sedimented at 3,000 × *g* for 15 min, and resuspended in a small volume of PBS. The flagella were removed by homogenization of the bacterial suspension for 1 min in a mechanical blender (Waring Products, Manchester, United Kingdom), and the bacterial cells were then removed by centrifugation at 3,000 × *g* for 15 min. The flagella were then sedimented by high-speed centrifugation, acid treated, precipitated with ammonium sulfate, and finally resuspended in distilled water and stored at −20°C.

ELISAs. In preliminary experiments, four different ELISA formats were compared for the detection of SEF14 antibodies by using panels of chicken sera from *S. enteritidis*-infected, *S. typhimurium*-infected, and uninfected 3-week-old specific-pathogen-free (SPF) chicks and 16-week-old pullets. The four assays investigated were (i) an indirect ELISA with purified SEF14 fimbriae (SEF14 ELISA), (ii) a double-antibody sandwich indirect ELISA (SEF14-DAS ELISA) with MAb SEF14-9 (15), (iii) an SEF14 blocking ELISA, and (iv) an SEF14-DAS blocking ELISA. The SEF14-DAS ELISA was selected for further study because of its high degree of specificity (>90%) when it was applied to the panel of chicken sera from birds infected with different *Salmonella* serotypes. In all tests, serum samples were diluted 1 in 400 and egg yolks were diluted 1 in 200 before testing by ELISA.

(i) SEF14-DAS ELISA. MAb SEF14-9 (500 ng per well) was adsorbed onto wells of polystyrene microtiter plates in carbonate buffer (pH 9.6) for 18 h at 4°C. The wells were then blocked with 4% (wt/vol) casein for 1 h at 37°C, and an optimum dilution of purified SEF14 antigen was added to each well for 2 h at 37°C. The wells were then washed in PBS containing 0.05% (vol/vol) Tween 20 (PBST), and optimal dilutions of test chicken sera or egg yolk in PBST with 1% (wt/vol) dried skim milk were added to the wells for 1 h at 37°C. The wells were washed again, and a chicken antiglobulin conjugated to alkaline phosphatase was added to the wells for a further 1 h at 37°C. Antibody binding was detected by the addition of the chromogenic substrate *p*-nitrophenyl phosphate, and the optical densities were recorded at 405 nm after 30 min of incubation at 20°C.

(ii) LPS ELISA and FLAG ELISA. The indirect ELISA with LPS (LPS ELISA) and indirect ELISA with purified flagellin (FLAG ELISA) extracted from *S. enteritidis* were carried out as described previously (4, 8).

Experimental infections. (i) Infection of 1-day-old chicks. Five groups of SPF white leghorn chicks (five or six per group) were hatched and reared in separate sterile isolators. At 3 days of age, birds in the first four groups were orally infected with 0.1 ml of buffered peptone water (BPW) containing 10^8 CFU of either *S. enteritidis* LA5, *S. enteritidis* 11146/93, *S. panama* 10173/93, or *S. typhimurium* 2391N. The last group served as an uninfected control group, and the birds were given 0.1 ml of BPW. Every week for 6 weeks blood samples were taken from each bird and were stored at −20°C.

(ii) Infection of hens. Eighteen-week-old SPF hens were similarly divided into five groups, and each group was placed within a separate pen, with birds housed in individual cages. The chickens were orally infected with the same salmonella strains as the 3-day-old chicks, except that the inoculating dose was 10^9 CFU per bird.

In another experiment, 70 18-week-old Isabrown pullets were supplied from a commercial flock where birds tested negative for salmonellae at 4 and 14 weeks of age. On arrival, the birds were individually tagged and blood and cloacal swab specimens were taken from all the birds. At 20 weeks of age, 35 birds were given orally a 1-ml 10% (wt/vol) sodium bicarbonate solution; this was followed by the administration of 4×10^8 CFU of *S. enteritidis* LA5. The remaining birds in each group were left to mix with the infected birds and are referred to as the "in-contact" group. A control group of birds was mixed with hens infected with 5×10^8 CFU of *S. typhimurium* 2391N. Every week for 4 weeks blood samples were taken from each bird. Eggs were collected in two batches per week, and the shell and egg contents were cultured for salmonellae.

Field samples. Serum samples were obtained from six commercial flocks (two layer and four broiler breeders) and were examined for antibodies to SEF14, LPS, and flagellin as described above.

Bacteriological and postmortem examinations. Feces or cloacal swab specimens were taken at weekly intervals from all experimentally infected birds. They were examined for the presence of salmonellae by inoculation into BPW; this was followed by selective enrichment in selenite broth and then direct plating onto brilliant green and Rambach agars. At the end of the experiments, the birds were killed by cervical dislocation and postmortem examinations were performed. Samples of liver, spleen, reproductive tract, and intestine (ileocecal junction) were homogenized and inoculated into selenite broth; this was followed by direct plating onto brilliant green and Rambach agars for the detection of salmonellae.

RESULTS

Experimental infections. One-day-old chicks infected with salmonella strains survived throughout the experiment, with a few birds showing a transient mild diarrhea during the first few days after infection. All infected chicks intermittently excreted salmonellae and no cross-infection was observed. At the postmortem examination (42 days postinfection), salmonellae were isolated from the spleens, livers, and ceca of all of the infected chicks.

Infected hens remained healthy and, unlike the chicks, did not develop a transient diarrhea, although they all excreted salmonellae during the experiment. At the postmortem examination (42 days postinfection), salmonellae were isolated only from the ileocecal junction of infected birds.

Serological responses of chicks infected at 1 day of age. The SEF14, *S. enteritidis*-derived LPS, and *S. enteritidis*-derived flagellin serum antibody responses are presented in Fig. 1. Only birds infected with *S. enteritidis* strains produced antibodies to SEF14. The SEF14 response was reproducible and was first detected at 14 days after infection, peaked at day 21, and persisted for at least 42 days postinfection (Fig. 1a). In contrast, chicks infected with *S. enteritidis* and *S. panama* produced antibodies to LPS, but only after 21 to 28 days following infection (Fig. 1b). The flagellar response was very weak and was not detected until day 42 postinfection, but like the SEF14 response, the flagellar response was confined to chicks infected with *S. enteritidis* (Fig. 1c). Uninfected control birds tested negative by all of the ELISAs (optical densities, <0.2).

Serological responses of hens infected at 18 weeks of age. The SEF14, *S. enteritidis*-derived LPS, and *S. enteritidis*-derived flagellin serum antibody responses are presented in Fig. 2. Only hens infected with *S. enteritidis* strains produced antibodies to SEF14 (Fig. 2a). However, the response was very low and variable, and 3 of the 10 *S. enteritidis*-infected hens failed to elicit SEF14 antibodies throughout the 6-week period following infection. In contrast, hens infected with the different *Salmonella* serotypes all produced rapid and high antibody responses to LPS and flagellar antibodies (Fig. 2b, c). LPS and flagellar antibodies were detected 7 days after infection and persisted for the 6-week period of study. Serological responses to LPS and flagellin were similar regardless of the *Salmonella* serotype used to infect the hens. Antibodies to SEF14, LPS, or flagellin were not detected in uninfected hens.

To mimic more accurately field situations, the SEF14 responses of uninfected hens in contact with birds infected with *S. enteritidis* or *S. typhimurium* were investigated over a 28-day period by using the SEF14-DAS ELISA, and the results are presented in Fig. 3. The cutoff value between positive and negative samples in the SEF14-DAS ELISA was determined by the examination of sera taken from hens prior to infection. The positive/negative value (0.3) was taken as the mean optical density plus 3 standard deviations. Salmonellae were isolated from all of the in-contact hens on at least one sampling occasion during the experiment. After 1 and 4 weeks of infection, 90 and 85% of the hens in contact with *S. enteritidis*-infected hens, respectively seroconverted to SEF14, with optical densities at a serum dilution of 1 in 400 ranging from 0.3 to >1.0 (Fig. 3). In contrast, none of the hens in contact with *S. typhi-*

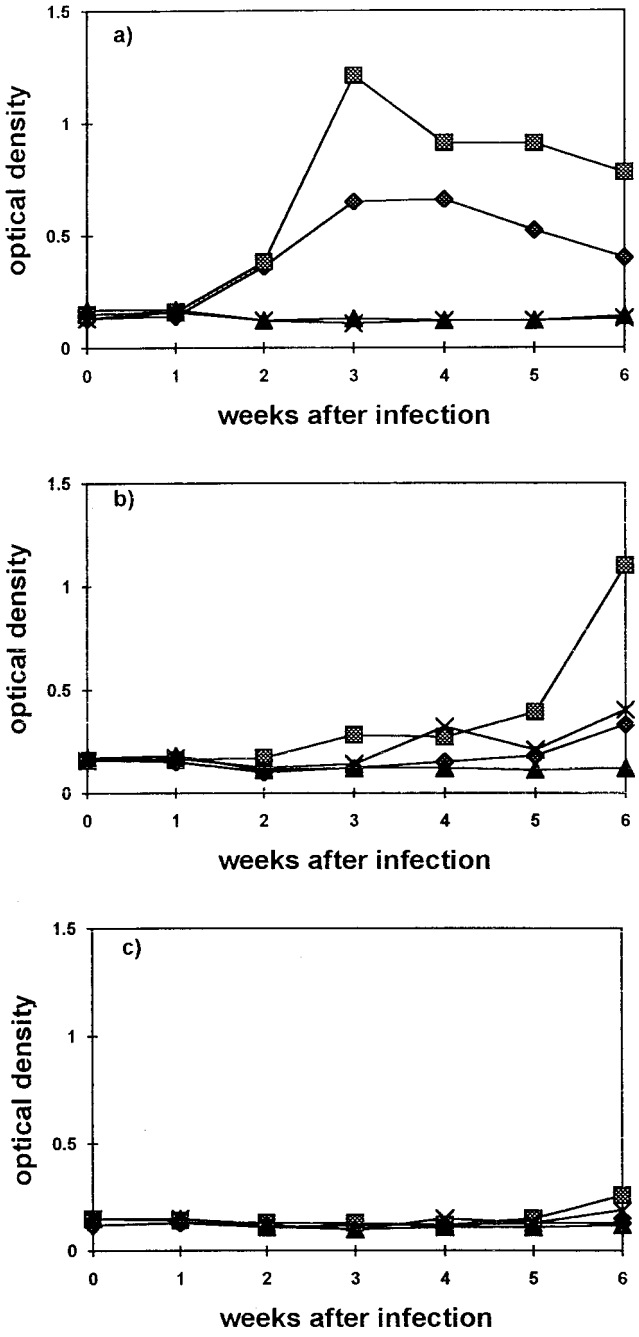

FIG. 1. Mean optical density values in serum at 1 in 400 dilution per group of experimentally infected 1-day-old SPF chicks in the SEF14-DAS ELISA (a), the *S. enteritidis* LPS ELISA (b), and the *S. enteritidis* FLAG ELISA (c). Symbols: ▨, *S. enteritidis* phage type 8; ◆, *S. enteritidis* phage type 4; ▲, *S. typhimurium*; ×, *S. panama*.

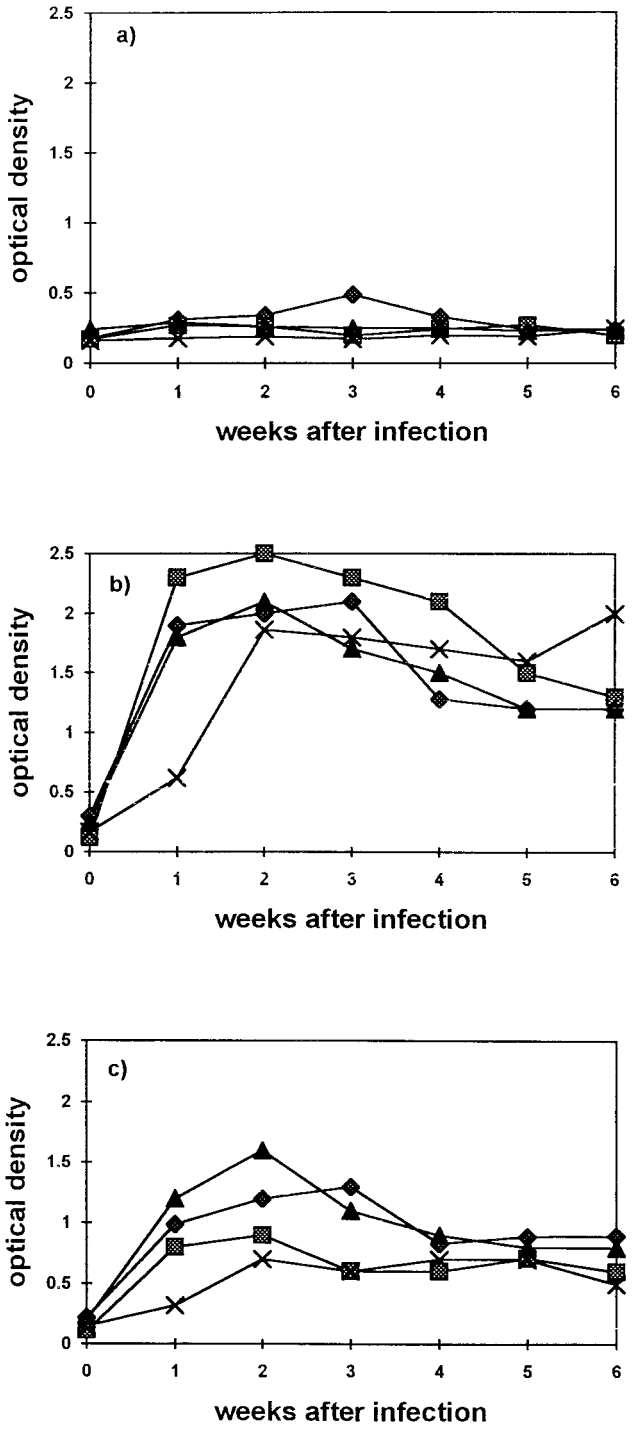

FIG. 2. Mean optical density values in serum at 1 in 400 dilution per group of experimentally infected 18-week-old SPF hens in the SEF14-DAS ELISA (a), the *S. enteritidis* LPS ELISA (b), and the *S. enteritidis* FLAG ELISA (c). Symbols: ▨, *S. enteritidis* phage type 8; ◆, *S. enteritidis* phage type 4; ▲, *S. typhimurium*; ×, *S. panama*.

murium-infected, hens produced SEF14 antibodies at any time during the 28-day period. All of the hens produced consistently high LPS responses, regardless of the *Salmonella* serotype used to infect the birds (data not shown).

Antibody responses in egg yolks. More than 400 eggs laid by hens from the infected and in-contact groups were collected during the 4-week period, and the egg yolks were separated and examined for SEF14 antibodies by the SEF14-DAS ELISA (Fig. 4). SEF14 responses were detected only in the *S. enteritidis*-infected groups. Seventy percent of the eggs laid from the infected and in-contact groups during the first week following exposure to *S. enteritidis* did not contain any SEF14 antibodies in their yolks, as detected by the SEF14-DAS ELISA. The remainder had low SEF14 responses of between

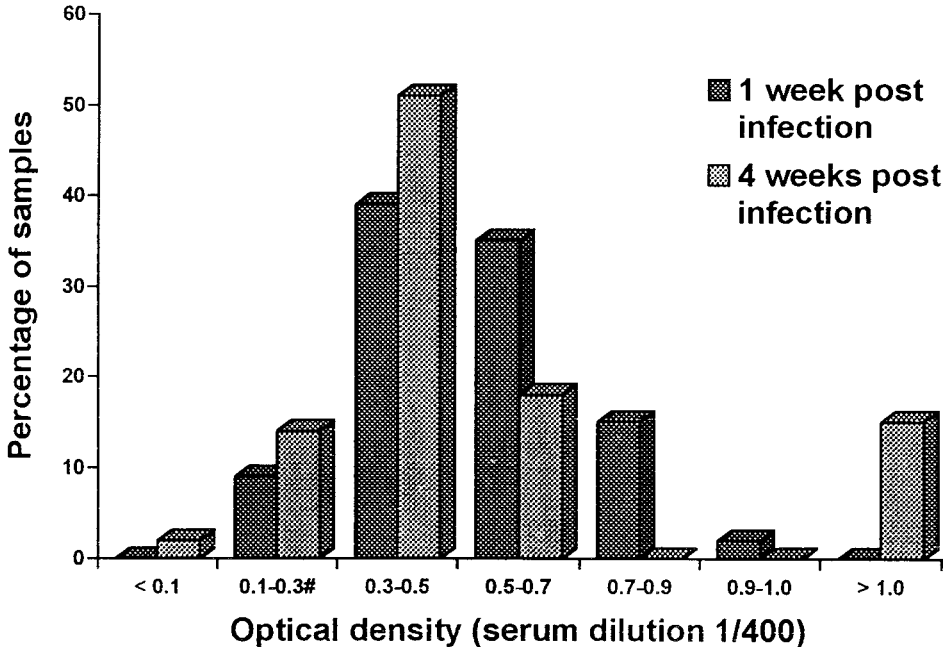

FIG. 3. SEF14 antibody response in serum of 18-week-old chickens in contact with *S. enteritidis*-infected chicken. Thirty-four hens were confirmed to be positive for *S. enteritidis* by culture of cloacal swab specimens. #, negative/positive cutoff value for serum samples.

0.3 and 0.7 optical density unit (Fig. 4). After a further 28 days, approximately 70% of the eggs laid had no detectable SEF14 antibody; however, 25% of the eggs contained high levels of SEF14 antibody, with optical densities in the SEF14-DAS ELISA being greater than 1.0 (Fig. 4).

Antibody responses in naturally infected chickens. The SEF14-DAS ELISA was used to test sera from six chicken flocks known to be infected or uninfected with *S. enteritidis*. The SEF14-DAS ELISA results were then compared with those obtained by the LPS ELISA and the FLAG ELISA (Table 1).

A complete correlation between culture-positive flocks and the presence of SEF14, LPS, and flagellar antibodies was obtained, although the ratio of positive to negative samples was dependent on the type of ELISA used (Table 1). In the most extreme case, only two serum samples were positive in the FLAG ELISA, whereas 37 and 30 serum samples that were positive in the LPS ELISA and the SEF14-DAS ELISA, respectively (Table 1, flock D). All of the ELISAs were negative with sera from flock F, the chickens in which were culture negative and were considered to be free of *S. enteritidis* infection.

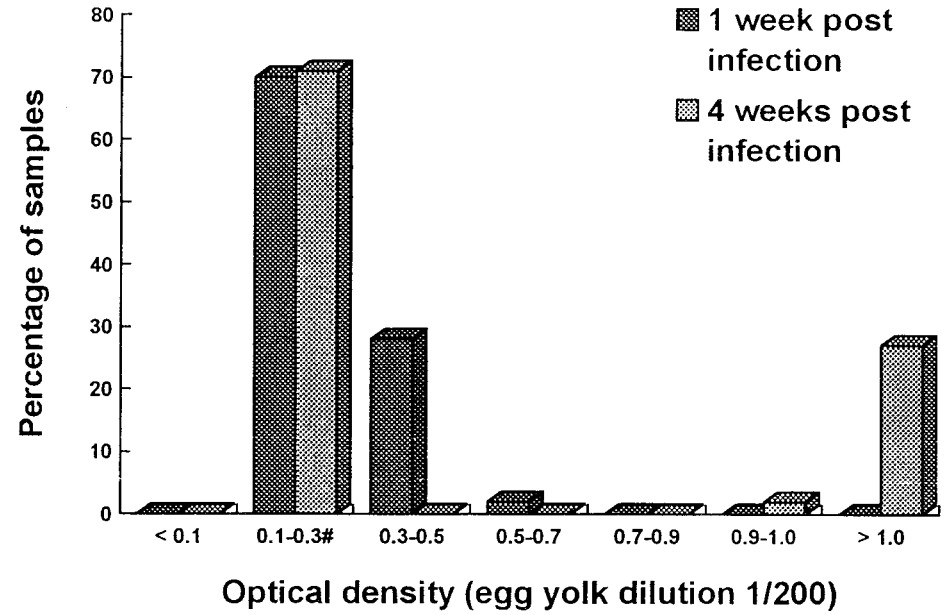

FIG. 4. SEF14 antibody response in eggs from *S. enteritidis*-infected hens. A total of 410 egg yolks were examined. #, negative/positive cutoff value.

TABLE 1. Serological and bacteriological examination of *S. enteritidis* infection in chicken flocks

Flock, flock type[a]	No. of samples positive			Culture result[b]
	SEF14-DAS ELISA	LPS ELISA	FLAG ELISA	
A, LB (50)	48	42	40	+
B, BB (29)	29	28	23	+
C, LB (40)	30	37	2	+
D, BB (30)	19	14	15	+
E, BB (210)	54	105	ND[c]	+
F, BB (30)	0	0	0	−

[a] LB, layer-breeder; BB, broiler-breeder; values in parentheses are total number of samples tested.
[b] +, *S. enteritidis* isolated from flock; −, *S. enteritidis* not isolated from flock.
[c] ND, not determined.

DISCUSSION

S. enteritidis is the only serotype isolated from poultry that expresses SEF14 fimbriae (15), and this characteristic has recently been exploited in the development of latex tests for the specific identification of cultured *S. enteritidis* isolates (13). Thus, SEF14 is an obvious candidate antigen for use in the serological detection of flocks infected with *S. enteritidis.*

As predicted, the SEF14 response was highly specific to birds infected with *S. enteritidis*. However, there were marked differences in the SEF14 response between chicks and laying hens that were experimentally infected with *S. enteritidis*, with the older birds producing a weaker but nevertheless specific response. This was in direct contrast to the LPS responses, which were delayed and variable in chicks but which were rapid, reproducible, and strong in laying hens, a phenomenon that has been described before (20). It was also surprising to find that the flagellar response in adult birds was not specific and occurred among birds infected with *S. typhimurium*. This observation, however, is in agreement with that of van Zjiderveld et al. (19), who suggested that cross-reactions were due to common epitopes on the different flagellins produced by the salmonellas in assays detecting a range of flagellar epitopes. Alternatively, it cannot be ruled out that small amounts of contaminating LPS in the flagellin preparations contributed to the nonspecific responses.

The lack of a reproducible SEF14 response in experimentally infected layers is not fully understood. However, birds infected with multiple doses of *S. enteritidis* and birds infected parenterally produce stronger and more reproducible SEF14 antibody responses (unpublished data). It is possible that the degree of invasion and persistence of *S. enteritidis* helps to dictate the serological response to SEF14 and chicks that are more susceptible to infection produce a stronger, more reproducible SEF14 response.

The relatively poor SEF14 antibody responses seen in experimentally infected layers prompted further investigations with sera and eggs either from chickens in contact with infected birds or from naturally infected flocks. The results clearly indicate that in the ELISA used to differentiate infected from uninfected flocks, complete agreement between culture and SEF14 antibody status was observed.

The results of the SEF14-DAS ELISA suggest that it will be possible to combine the characteristics of the high degrees of sensitivity of LPS ELISAs with the high degrees of specificity of ELISAs based on flagellar epitopes for the mass screening of flocks for *S. enteritidis*. The high degree of specificity will assume greater significance if the prevalence of *S. enteritidis* in chickens falls to very low levels because of the active control measures adopted in various countries.

In the last few years, live and killed salmonella vaccines for chickens have been produced and have been shown to be effective in reducing the shedding of *S. enteritidis* (3, 17). They may be precluded from control programs because of their interference with serological tests used to detect natural infection. Nevertheless, their use is now gradually increasing, which has resulted in a reduction in serological screening because currently available tests cannot discriminate vaccinal antibody responses from natural infection. SEF14 is not considered a virulence determinant of *S. enteritidis* in chickens (16). It is therefore likely that the specific deletion of *sefA* (the structural gene encoding SEF14) will not compromise the efficacy of a vaccine. If this is the case, vaccinal responses devoid of SEF14 antibodies will be distinguished from the production of SEF14 antibodies in natural infections.

This is the first report describing the use of a fimbrial antigen in the serodiagnosis of salmonella infections. It is quite likely that other *Salmonella* serotypes express their own unique fimbriae and, together with SEF14, will become candidate serodiagnostic antigens for the future detection of salmonella infections in animals and humans.

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