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The influence of age on the response of SPF hens to infection with *Salmonella enteritidis* PT4

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SUMMARY

When Specific Pathogen-Free hens were infected with *Salmonella enteritidis* PT4 by direct administration into the crop, the age of the bird at infection was found to have an effect on both pathogenesis and antibody response. Birds at 20 weeks of age showed no adverse signs and developed high titres of antibodies of the IgM class, while those which were 1 year old at infection developed relatively little antibody and had acute septicaemia, with 6 of 10 birds either dying or having to be humanely destroyed. The implication of these results for the control of salmonella infections in poultry is discussed.

INTRODUCTION

Salmonella enteritidis phage type (PT) 4 is invasive in young chicks [1] and in some commercial broiler flocks significant levels of mortality have been recorded [2]. Chickens generally become more resistant to salmonella infection with age and it is possible to protect young chicks by the oral administration of cultured caecal contents from an appropriate mature donor bird [3]. Recent experimental studies demonstrated, however, that mature hens of 52–55 weeks of age could be infected by the direct administration of 100 cells of *S. enteritidis* PT4 into the crop [4]. Infection was systemic and the organism was isolated from viscera, including reproductive tissue. In parallel studies, point-of-lay pullets at approximately 18 weeks of age remained largely unaffected although the organism was isolated from egg contents [4]. This implied that the response of the birds to oral challenge with *S. enteritidis* PT4 may be governed, to some extent, by their age at the time of infection. Thus, while the organism was invasive in the older birds and was isolated from the viscera of 5 of 12 birds in the first 2 weeks after infection the tissues of 16 of the younger birds were salmonella-negative [4].

The above studies were performed using birds from commercial laying flocks. There is the possibility that the two groups may have had differing exposures to

salmonellas prior to artificial infection with *S. enteritidis* PT4 and that this could have played some part in their responses to the organism. In an attempt to gain a clearer picture of the response of egg-laying hens to infection by the direct administration of *S. enteritidis* PT4 into the crop two groups of Specific Pathogen Free (SPF) hens, one at 20 weeks of age and the other at 52 weeks, were infected. In longitudinal studied lasting over 3 months faecal samples, egg shells and contents and viscera were examined for the presence of salmonellas. Note was made of the birds' feeding, egg-laying and behaviour and blood samples were taken at intervals and examined for antibodies against *S. enteritidis* lipopolysaccharide (LPS).

MATERIALS AND METHODS

Birds

Twenty, 20-week-old and twelve 52-week-old SPF White Leghorn hens, termed flocks SPF-I and SPF-II respectively, were used in the studies. They were housed in individual metal cages with wire mesh floors, which were cleaned and disinfected daily. The birds were fed pelleted, irradiated commercial feed and allowed to drink water *ad libitum*. Artificial light was provided on a 12 h on, 12 h off cycle.

Infection

In each experiment, following starvation for 24 h, the birds were inoculated in the crop by catheter with approximately 10^6 cells of *S. enteritidis* PT4 strain P125592. With the SPF-I birds, 18 were infected in this manner with two others being given peptone water only. These were kept as controls and were housed in the same room as the experimental birds. In the experiment with the SPF-II hens there were 10 experimental birds and two controls.

Bacteriological examinations

At purchase, cloacal swabs were taken. Following infection, eggs and faecal samples were collected daily. The former were stored at +4 °C and the latter at room temperature until microbiological examination.

The presence of salmonellas was determined using standard enrichment methods for faeces, cloacal swabs and viscera (see below), and previously published techniques [5] for egg shells and contents.

Necropsy

The birds in the first experiment (SPF-I) were killed 20 weeks after infection by inhalation of carbon dioxide. Specimens of liver, spleen, jejunum, blood, oviduct, ovule, ovary tissue, crop and caecal contents were taken for bacteriological examination. Portions of liver, ileum, caecum, oviduct and kidney were also taken for histological examination.

The 12 SPF-II birds either died or were killed at intervals between 8 days and 11 weeks after infection.

Histological examination

Specimens were fixed immediately after necropsy in 10% buffered neutral formalin, processed by standard methods and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin.

Measurement of antibodies against S. enteritidis

Lipopolysaccharide

Lipopolysaccharide (LPS) was prepared from *S. enteritidis* strain P132344 using hot-phenol [6]. Replicate aliquots of LPS, stored at -10 °C, were used for ELISA tests.

ELISA

Enzyme-linked immunosorbent assays were performed as described previously [6]. ELISA plates were coated with 0·1 μg LPS preparation in coating buffer and reacted with 100 μl serum diluted ($\times 500$) in phosphate buffered saline. Antibodies of the IgG class was detected using an alkaline phosphatase conjugated goat anti-chicken IgG antibody (Southern Biotechnology). Antibodies of the IgM class were detected by reacting plates with a goat anti-chicken IgM antiserum (Nordic Immunology Ltd), followed by an alkaline phosphatase conjugated rabbit anti-goat Ig antibody (Sigma Chemical Co.).

Salmonella whole-cell agglutinations

A 'pullorum antigen' obtained from Ministry of Agriculture Fisheries and Food, Weybridge, England, was used for serum agglutination reactions [6].

RESULTS

The influence of bird age of the course of S. enteritidis *infection*

All cloacal swabs taken from all birds prior to infection were negative for salmonellas.

There was a difference in the response to challenge with *S. enteritidis* in the two groups of birds. The younger, SPF-I birds appeared unaffected by the organism. None had diarrhoea and faecal carriage essentially ended after the first week post infection (Table 1). All 18 birds remained well and continued to behave and feed normally. All specimens of viscera collected 20 weeks after infection were salmonella-negative. None of 283 egg contents were positive for salmonellas although this may have been because most of the birds did not start laying until 37 ± 4 days post-infection. There was, however, a cluster of *S. enteritidis*-positive shells from eggs laid by 10 different birds including one of the control birds (No. 19) between 45 and 52 days after infection (Table 1).

In contrast, the 10 birds infected at 52 weeks of age (SPF-II) showed a severe response to challenge with *S. enteritidis* PT4. All those in the experimental group were dull and off feed from the fourth to fourteenth day after infection and developed acute and protracted diarrhoea. There was prolonged faecal carriage and 6 of 10 birds either died from an overwhelming salmonella septicaemia or its effects or had to be humanely destroyed (Table 2). *Salmonella enteritidis* PT4 was

Table 1. *Faecal carriage of and egg shell contamination with* Salmonella enteritidis *PT4 in SPF-1 pullets infected at 20 weeks of age*

Hen no.	Days post infection: 1	2	3	4	5	6	7	13	15	19	22	26	29	33	36	41	45	46	47	48	49	50	51	54	57	60	63	66
A1	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A2	F	·	·	F	F	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	·	·
A3	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·
A4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	S	·	·	·	·	·	·	·	·
A5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	S	·	·	·	·	·	·	·
A6	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	·	·
A8	F	·	·	F	F	F	·	·	·	·	·	·	·	·	·	·	·	S	S	·	·	·	·	·	·	·	·	·
A9	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	S	S	·	·	·	·	·
A11	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·
A12	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A13	F	·	·	·	F	F	F	·	·	·	·	·	F	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·
A14	·	·	·	·	F	F	·	·	F	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·
A15	F	F	F	F	F	F	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A16	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A17	F	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A18	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
A19*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·
A20*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

* Uninfected controls.
F, Faecal sample positive for *S. enteritidis*.
S, Egg shell positive for *S. enteritidis*.

Table 2. *Faecal carriage of* Salmonella enteritidis *PT4 in SPF-II hens infected by oral inoculation at 52 weeks of age*

Bird no.	Days post infection: 2	4	6	8	10	12	14	16	18	24	26	28	30	32	34
1	+	+	+	+	+	+	+	+	+	+	+	K			
2	+	+	+	+	−	+	+	+	−	−	−	−	−	−	−
3	+	+	+	+	+	+	+	+	+	+	+	+	D		
4	+	+	+	+	D										
5	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−
6	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−
7	+	+	+	+	+	−	−	+	−	+	+	−	+	−	−
8	+	+	+	+	+	+	−	+	−	−	+	+	−	+	K
9	+	+	+	+	+	+	+	+	+	+	+	K			
10	+	+	+	+	+	+	+	+	+	+	−	K			

K, Killed; D, died.

Table 3. *The isolation of* Salmonella enteritidis *PT4 from the viscera of SPF-II hens infected at 52 weeks of age*

Bird no.	Days post infection killed/died	*Salmonella enteritidis* isolated from: Ileum/jejunum	Blood	Liver	Spleen	Bile	Ovary	Oviduct	Crop	Caecum
B4	8	+	+	+	+	+	+	+	+	+
B1	27	−	−	+	+	−	NT	−	NT	+
B9	28	+	−	−	−	NT	+	−	NT	+
B10	28	−	−	+	NT	NT	−	−	−	−
B3	29	+	−	−	−	−	−	−	NT	+
B8	35	−	−	−	−	−	−	−	NT	−

NT, Not tested.

also isolated from faecal samples from both of the control birds. With bird No. 11, 10 samples were positive between 6 and 16 days after infection of the experimental birds. One sample, taken from bird No. 12 6 days after infection was salmonella-positive. One egg out of 223 was positive for *S. enteritidis* PT4 in the contents and the organism was isolated from the shells of 8 eggs laid by 4 birds between 3 and 14 days after infection.

Salmonella enteritidis PT4 was also isolated from the viscera of some of the birds at post mortem (Table 3).

Necropsy and histopathological findings

SPF-I birds

No macroscopic or microscopic lesions were observed at necropsy in either infected or control birds.

SPF-II birds

At necropsy of bird B4, which died 8 days after infection, there was widespread suppurative peritonitis, particularly involving the surfaces of the liver and serosa of the intestines. The intestines and caeca were distended by watery contents.

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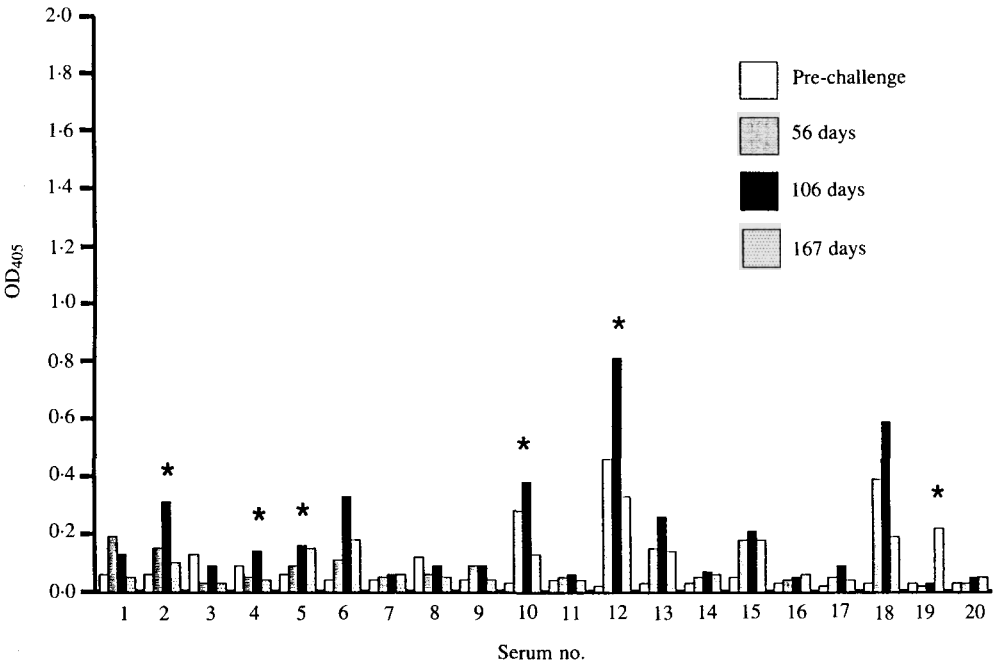

Fig. 1. Histogram plotting reaction of serum antibodies (IgG) with *S. enteritidis* LPS by ELISA. Analysis of four sera taken from each bird from flock SPF-I, during the course of experimentation (key indicates number of days post-challenge birds were sampled) showed an increase in IgG antibodies in some birds, with highest titres detected in samples taken 106 days post-challenge. Five infected birds and one control chicken contained agglutinating antibodies 167 days post-challenge (*).

Birds killed or dying at 1 month or later had abdominal fibrous adhesions indicative of an earlier peritonitis and there were numerous small pale foci in the liver and kidneys. At this stage intestinal contents had returned to normal.

Histopathological examination of organs from bird B4 showed numerous large areas of necrosis of parenchymatous cells in the liver, with only a minimal macrophage response at the periphery of the lesions. Similar foci were also scattered throughout the lungs and kidneys. The lamina propri and epithelium of the ileum and caeca were heavily infiltrated by neutrophils and the submucosa was oedematous. Acute inflammatory changes were also present on the serosal surfaces of the intestines and oviduct. The liver of birds killed or dying 1 month post infection contained scatter foci of lymphocytes and macrophages, and the kidney lesions were now represented by fibrosis of the cortex and medulla and sequestered aggregates of neutrophils. Many tubules and collecting ducts contained casts, neutrophils and mineral deposits. The mucosa of the oviduct was normal in all birds.

The organs of control birds showed no abnormalities.

Serology

Sera prepared from blood samples taken from both flocks, prior to bacterial challenge, were found to contain very low levels of antibodies of both IgG and IgM classes, with ELISA values of 0·1 (A_{405}) or less (Figs. 1–4). When the SPF-I birds

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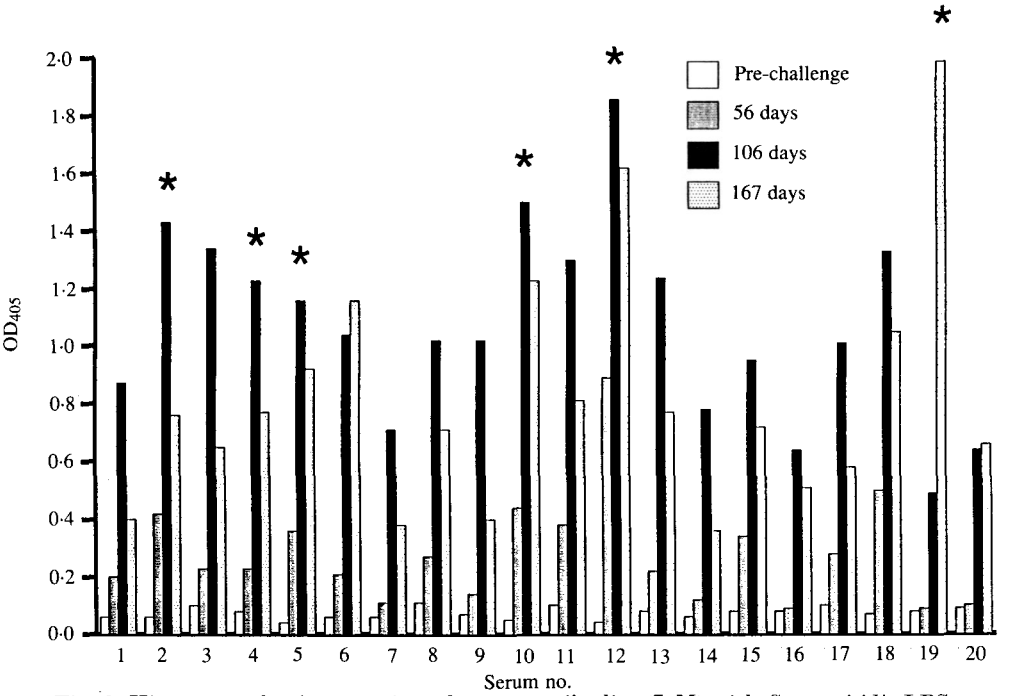

Fig. 2. Histogram plotting reaction of serum antibodies (IgM) with *S. enteritidis* LPS by ELISA. Analysis of four sera taken from each bird from flock SPF-I, during the course of experimentation (key indicates number of days post-challenge birds were sampled) showed an increase in IgM antibodies in all 18 birds, with highest titres detected in samples taken 106 days post-challenge. Five infected birds and one control chicken contained agglutinating antibodies 167 days post challenge (*).

were sampled at 56 days post challenge, only sera from hens 10, 12 and 18 contained noticeably higher levels of IgG antibodies (Fig. 1).

In contrast, all samples from the challenged birds in this group contained high levels of IgM antibody with titres considerably higher than in the samples from pre-challenge birds (Fig. 2). At 106 days post-challenge, birds 2, 6, 10, 12 and 18 showed a further increase in levels of IgG antibodies (Fig. 1), and all challenged birds showed a considerable increase in the levels of IgM antibodies (Fig. 2). At this stage, blood samples from the two control birds were also shown to contain IgM antibodies to *S. enteritidis* (Fig. 2). Birds sampled prior to the termination of experimentation (167 days) had lower levels of both IgG and IgM than detected at 106 days post-challenge (Figs. 1, 2). At termination, birds 2, 4, 5, 10 and 12 had antibodies agglutinating the pullorum antigen (Figs. 1 and 2). Surprisingly, the sera from control birds 19 and 20 were also shown to contain high levels of IgM antibodies, with bird 19 and 20 giving an ELISA value of nearly 2·0. Bird 20 was also found to have agglutinating antibodies (Fig. 2).

ELISA tests using sera prepared from SPF-II hens 27–35 and 74–86 days post-challenge did not show noticeable increases in levels of either IgG or IgM antibodies (Figs. 2, 3). Bird 9 showed an increase in IgG antibodies at 27 days post-challenge, but developed a fatal infection before sampling at 74 days (Fig. 3). Bird 2 showed an elevated level of IgM antibodies by 74–86 days post-challenge (Fig. 4).

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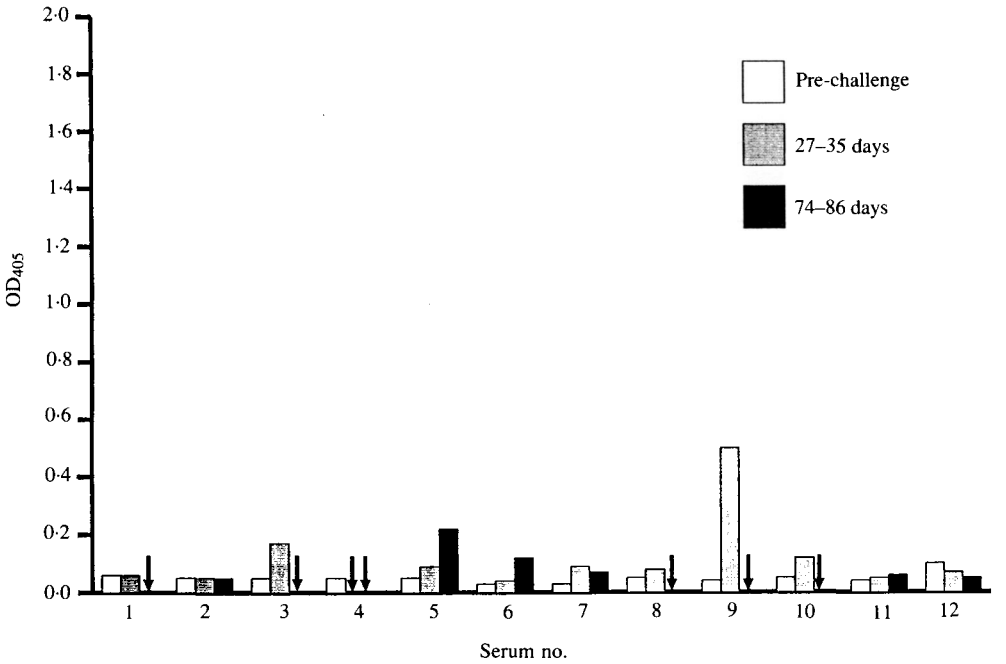

Fig. 3. Histogram plotting ELISA results obtained by reacting chicken sera from flock SPF-II with *S. enteritidis* LPS. Three sera were taken from each bird during the course of experimentation (key indicates number of days post-challenge birds were sampled). Infected birds (1–10) failed to amount a significant IgG antibody response when compared to the levels of this antibody class in control chickens (11 and 12). Arrows indicate absence of serological data due to fatality.

DISCUSSION

The results presented in this paper support earlier observations [4] and demonstrate that mature chickens can readily be infected with *S. enteritidis* PT4. It would also appear that, as with commercially reared birds, the older (52 weeks) SPF-II hens were more adversely affected by the organism. They also failed to elaborate significant levels of antibodies. Thus, while birds at 20 weeks apparently remained well and produced high levels of IgM antibody, 6 of 10 birds infected at 52 weeks either died or had to be humanely killed. As far as was practically possible the two groups of birds were kept in identical conditions, fed the same irradiated feed and given the same dose of the same strain of *S. enteritidis* PT4. The differences in response to infection are therefore more likely to have resulted from differences between the two groups of birds rather than variations in their environment or treatment.

A possible explanation for the phenomena observed in this and earlier studies might relate to changes to the immune status of the older birds which could have occurred as a consequence of fatigue associated with intensive laying. Calcium depletion in the SPF-II hens might also have had an effect. The younger birds could have been protected by oestrogens.

The use of competitive exclusion has been shown to protect chicks against infection with *S. enteritidis* PT4 whether the organism was administered directly

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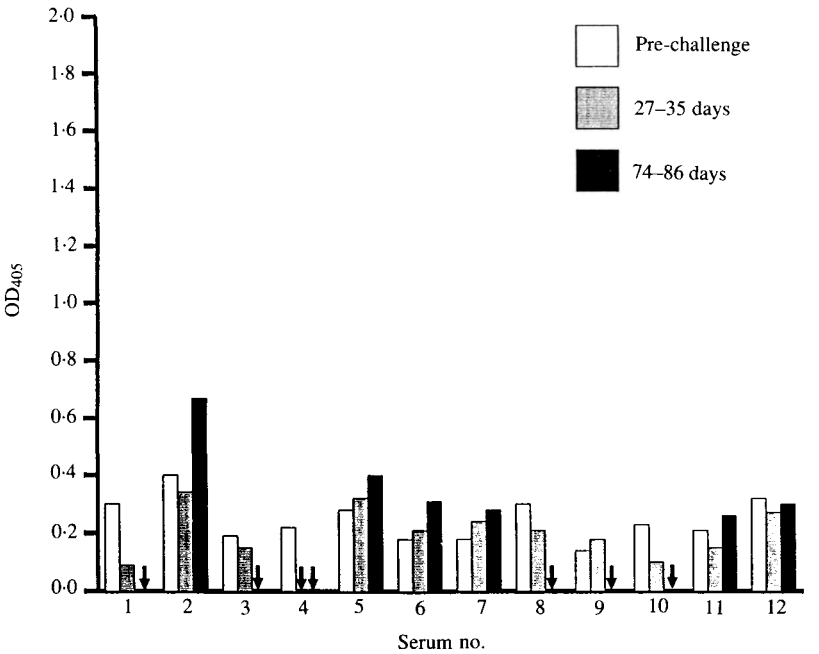

Fig. 4. Histogram plotting ELISA results obtained by reacting chicken sera from flock SPF-II with *S. enteritidis* LPS. Three sera were taken from each bird, during the course of experimentation (key indicates number of days post-challenge birds were sampled). Infected birds (1–10) failed to amount a significant IgM antibody response when compared to the levels of this antibody in control chickens (11 and 12). Arrows indicate absence of serological data due to fatality.

into the crop, via drinking water or infected seeder birds [7]. This technique clearly has an important role as an anti-salmonella control measure, particularly in the broiler chicken industry. The results presented in this paper might imply that the protective nature of the gut flora declines as the bird ages and this is being investigated. Birds at 60 weeks of age, however, are used regularly to supply caecal contents for use in competitive exclusion (Mead, personal communication). The main purpose of the work reported in this paper was to examine the influence of bird age on the response to *S. enteritidis* PT4. To ensure infection, the dose used exceeded that likely to be experienced by birds kept under commercial conditions and the results should not necessarily be taken as a criticism of competitive exclusion. What they do show is that some, as yet, unknown factor causes older hens to be much more susceptible to infection with *S. enteritidis* PT4.

The detection of antibodies in sera from the control birds (19 and 20) from flock SPF-I, and the presence of viable *S. enteritidis* on the shell of one egg from bird 19 indicates that these birds had been infected accidentally during experimentation. Control birds were kept in the same room as challenged birds but considerable care was taken to avoid cross contamination. Both the older birds, however, became infected suggesting that the infective dose was probably quite small and/or the organism was highly contagious under the experimental conditions used. How this relates to the commercial chicken flocks has yet to be established.

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Whatever the explanation for the different responses in the chickens it demonstrates the need to protect chickens against *S. enteritidis* throughout their life. The major attribute of this salmonella serovar is the ability to be transmitted vertically from infected parent flocks as demonstrated by occasional high mortality in young broiler chicks [2], its isolation from ovaries and ova from a commercial laying flock [8] and from egg contents [5]. Thus, the ultimate control of infection must be linked to the identification and eradication of infected parent and grandparent flocks. *Salmonella enteritidis* PT4 has been isolated from poultry feed and investigations in Northern Ireland implicated contaminated feed as the source of infections in some broiler-breeder flocks [9]. The results from this present and earlier [4] studies could be taken to suggest that if hens were to receive contaminated feed towards the end of their commercial laying period, systemic infection might result with the concomitant infection of chicks and/or contamination of egg contents.

Salmonella enteritidis PT4 can be isolated from both the yolk and albumen of intact shell eggs [5]. Its presence in the former site is presumably as a result of infection of the ovary while contamination of the albumen may result from infection of the upper oviduct. Contamination of egg shells with salmonellas is usually thought to be the result of faecal carriage [10]. While this, or the environment, could clearly have been the source in the study reported here, the apparent lack of relationship between presence of *S. enteritidis* PT4 in faeces and on egg shells with the SPF-I birds (Table 1) raises other possibilities. The organism might either be able to colonize the shell gland or infections in the kidneys could result in shell contamination at lay. These questions and many others about this important human pathogen, can only be answered by detailed investigation of naturally infected hens.

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