Epidemiol. Infect. (1992), **108**, 389–396
Printed in Great Britain

Persistence of contamination of hens' egg albumen *in vitro* with *Salmonella* serotypes

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(*Accepted 12 December 1991*)

SUMMARY

A study was made of the persistence of different *Salmonella* serotypes in hens' egg albumen *in vitro* at 4, 20 and 30 °C. The majority of serotypes remained viable but did not increase in numbers at 20 and 30 °C for 42 days. At 4 °C many of the serotypes died out.

The addition of ferric ammonium citrate on the 42nd day of incubation induced multiplication of organisms incubated at 20 and 30 °C, but not at 4 °C. The pH and glucose concentration of the albumen diminished only when heavy growth occurred.

Salmonella enteritidis remained viable on the air cell membrane *in vitro* for 17 days at 4, 20 and 30 °C. Thirty percent of the organisms also remained motile in albumen for 42 days at 25 °C and up to 5% of the cells remained motile for up to 20 days at 4 °C.

INTRODUCTION

Salmonella enteritidis, predominantly phage type 4 (PT4), has been associated with outbreaks of salmonellosis in which eggs and egg products were the vehicles of transmission [1]. There are two possible routes of salmonella contamination of the egg contents. In one, contamination occurs before oviposition [2, 3]. The incidence is about 0·6% with naturally contaminated eggs, the level of contamination is low and the albumen is more frequently contaminated than the yolk [4]. In the other, organisms on the shell are translocated along the pore canals and lodged on the underlying shell membranes. Translocation may occur during egg washing if the wash water is cooler than the egg [5–7]. The temperature differential causes a slight negative pressure because the reduction in volume of the contents is greater than that of the shell [5]. This results in a small amount of contaminated water being sucked into an egg [5, 8]. In this instance the cuticle enveloping the outer surface of the calcite shell is the main impediment to water and bacterial translocation along the pore canals [9]. The immature cuticle on eggs at or for a few minutes following oviposition appears to be an ineffective barrier [9, 10]. Indeed, this may well explain the higher incidence of contamination of the contents of eggs laid on the floor of poultry houses over those laid in nest boxes [11–13]. Such studies have shown that a range of organisms gains access to the contents of eggs infected in this manner.

Many factors influence the behaviour of organisms that contaminate the shell membranes post trans-shell infection [14]. A recent study [15] showed that the storage temperature had a profound selective action on the members of a consortium of bacteria (*Pseudomonas putida*, *Staphylococcus xylosis*, *Enterococcus faecalis*, *Escherichia coli* and *Salmonella enteritidis* PT4) seeded on the membranes. *Pseudomonas putida* became the dominant organism in the shell membranes and eventually in the albumen of eggs stored at 4, 15 and 20 °C. In contrast, *Salmonella enteritidis* dominated the infection of both the shell membrane and contents of eggs stored at 37 °C. Clay and Board [16] noted that a pure culture of *S. enteritidis* was confined to the shell membranes of eggs stored at 4 °C. With storage at ambient temperature, there was a progressive increase in the number of eggs containing $> 1{\cdot}0 \times 10^7$ salmonellas per ml of albumen. They surmised that the salmonellas retained in the membrane and the initial invaders of the albumen did not grow because of the antimicrobial properties of the latter [14, 17] and that the large populations arose from the growth of contaminants which made contact with the yolk. Recently Humphrey and colleagues [4] concluded that contaminants which gain access to the albumen surrounding the yolk of eggs stored for 2–3 weeks grow on nutrients that have diffused from the yolk across the ageing vitelline membrane. It is evident that further work needs to be done to resolve these conflicting interpretations. The present study, which is a prelude to such investigations, examines the hypothesis that following trans-shell or oviducal infection of infertile hens' eggs the initial invaders of the albumen would not proliferate sufficiently to cause the gross contamination noted by Clay and Board [16].

MATERIALS AND METHODS

Eggs

Eggs (size 4, approx. 58 g) less than 2 days old were purchased from a local producer/retailer and stored for less than 2 days before use. Eggs were assumed to be salmonella-free at purchase. Eggs from the same source were used in other studies in which endogenous salmonellas would have been detected. None was found to be contaminated.

Cultures

The sources of the cultures are given in Table 1. These were stored on Dorset egg agar (Oxoid Ltd) at 4 °C and subcultured every 3 months. For experimental purposes, an overnight culture in nutrient broth (Lab M, incubated at 37 °C) was spun down (2000 g for 10 min), washed in saline (Lab M) and finally resuspended in the same medium and diluted such that 0·1 ml contained *c.* 10^3 organisms.

Persistence experiments

Eggshells were wiped with ethanol (70% v/v), cracked and the contents collected. Albumen and yolk were harvested and bulked separately. Seventy ml of blended albumen were dispensed into sterile containers (250 ml, Sterilin) and 0·1 ml of a cell suspension added. Duplicate samples were stored at 4, 20 or 30 °C and sampled regularly. Ferric ammonium citrate (BDH) was added (final concentration 0·008 mg/ml Fe^{3+}) on the 42nd day.

Viable counts were obtained by spreading 0·1 ml of an appropriate dilution on duplicate plates of Xylose Lysine Decarboxylase agar (XLD; Lab M) with overnight incubation at 37 °C. Presumptive salmonella colonies on XLD were confirmed serologically.

Initial and final glucose concentrations were determined (Boehringer Mannheim). The pH of the albumen was tested with Whatman indicator paper (range 1–14).

The in vitro *study of* S. enteritidis *on the inner shell membrane*

The air cells of eggs were located by candling. The shell was swabbed with 70% ethanol, a small hole drilled in the shell at this site and 0·1 ml of a *c.* 10^3 cell suspension injected onto the air cell membrane. The hole was sealed with paraffin wax and the eggs left at room temperature for *c.* 3 h. All the liquid was absorbed into the egg contents within 20 min of being applied to the shell membrane. Eggshells were then wiped with ethanol, cracked, the albumen separated and poured into sterile containers (250 ml Sterilin). The inoculated inner membrane of the air cell was excised from the shell and placed in the albumen.

The albumen was incubated at 4, 20 or 30 °C. Duplicate samples were tested for the presence of *S. enteritidis*.

Viable counts of the albumen were obtained on XLD. The membrane was plated directly onto XLD.

A motility index of organisms in albumen was obtained by comparing the number of motile cells and the number of non-motile ones. A minimum of 200 cells was counted per sample using × 1000 phase contrast microscopy.

RESULTS

The persistence of viable cells in albumen *in vitro* at 4, 20 or 30 °C was studied with 13 *Salmonella* serotypes and 13 phage types of *S. enteritidis* (Table 1). With the exceptions of *S. enteritidis* PT4 and *S. hadar*, and both on one occasion only, there was a progressive diminution in the viable counts in albumen at 4 °C such that our method of analysis failed (< 10 CFU/ml) to isolate organisms on the 42nd day of incubation. Microscopical examinations, which were done on every sampling occasion in one experiment with inoculated albumen at 4 °C, showed that up to 5% of *S. enteritidis* remained motile for 9–20 days. The addition of ferric ammonium citrate to albumen on the 42nd day of incubation at 4 °C did not induce multiplication of any of the *Salmonella* serotypes or phage types.

There was a spectrum of responses among salmonellas stored in unsupplemented albumen incubated at 20 or 30 °C. Eight of the 13 phage types of *S. enteritidis* (Table 1) multiplied sluggishly (generation time of days) at one or both of these temperatures (Fig. 1). When sluggish growth occurred neither the glucose content nor the pH of the albumen changed. It is evident from Fig. 1a that there was a very fast rate of growth following the addition of ferric ammonium citrate to albumen. When suspended in unsupplemented albumen at 25 °C, 30% of *S. enteritidis* PT4 cells were motile on the 42nd day of incubation, even though no demonstrable growth had occurred. The other phage types of this serotype merely persisted or their numbers diminished to undetectable levels in albumen at 20 or 30 °C. There was no demonstrable change in the glucose content or pH of the

Table 1. *The persistence of* Salmonella *serotypes in albumen* in vitro *with incubation at 4, 20 or 30 °C*

Salmonella serotype	Source*	Persistence at °C			Response to Fe^{3+}		
		4	20	30	4	20	30
enteritidis	A2†	+	+	+	ng	G	G
PT 4	A	−	g	g	ng	G	G
PT 4 cured	A	−	+	g	ng	G	G
PT 4a	B	−	g	(g)	ng	G	G
PT 1	B	−	g	g	ng	G	G
PT 6	B	−	g	(g)	ng	G	G
PT 13a	C	−	g	(g)	ng	G	G
PT 21	B	−	g	(g)	ng	G	G
PT 30	B	−	(g)	g	ng	G	G
PT 14b	B	−	(g)	(g)	ng	G	G
PT 5	B	−	(g)	(g)	ng	G	G
PT 12	B	−	(g)	+	ng	G	G
PT 8	C	−	(g)	−	ng	G	G
PT 24	B	−	+	+	ng	G	G
PT 23	B	−	+	−	ng	G	G
hadar	D2	+	+	(g)	ng	G	G
		−	(g)	(g)	ng	G	G
worthington	D	−	g	(g)	ng	G	G
waycross	E	−	(g)	+	ng	G	G
ohio	D	−	(g)	+	ng	G	G
brandenberg	E	−	+	+	ng	G	G
dublin	E	−	+	−	ng	G	G
infantis	E	−	+	−	ng	G	G
typhimurium	A	−	+	+	ng	G	G
montevideo	B	−	+	+	ng	G	G
senftenberg	D	−	+	+	ng	G	G
gallinarum	F	−	+	−	ng	G	G
pullorum	F	−	−	−	ng	G	G

* Source: A. Exeter PHLS: B. CVL (Weybridge): C. ex-egg USA: D. British United Turkeys: E. Bath University: F. Bristol University.

† All experiments done in duplicate. 2 indicates that 2 or more trials were conducted.

−, did not persist; +, persisted; (g), slight growth, generation time 13–19 days; g, growth, generation time 2–12 days; G, generation time 6–12 h.

albumen. Of the other serotypes, only *hadar* (Fig. 1b), *worthington*, *ohio*, and *waycross* multiplied in albumen at 20 or 30 °C. Again, however, the growth rate was sluggish and the results inconsistent. Thus, for example *S. hadar* merely persisted in two trials and grew in another (Table 1). In the latter instance the generation time was very long (> 13 days) such that there was only a 1000-fold increase in the population size during 42 days incubation of albumen at 20 °C. It only achieved a 100-fold increase at 30 °C.

Seven of the serotypes (*brandenberg*, *dublin*, *infantis*, *typhimurium*, *montevideo*, *senftenberg* and *gallinarum*) persisted but did not grow in albumen at 30 and/or 20 °C. The number of viable cells of *S. pullorum* diminished to undetectable levels at both of these temperatures.

Every strain of salmonella (Table 1) used in this study formed large populations

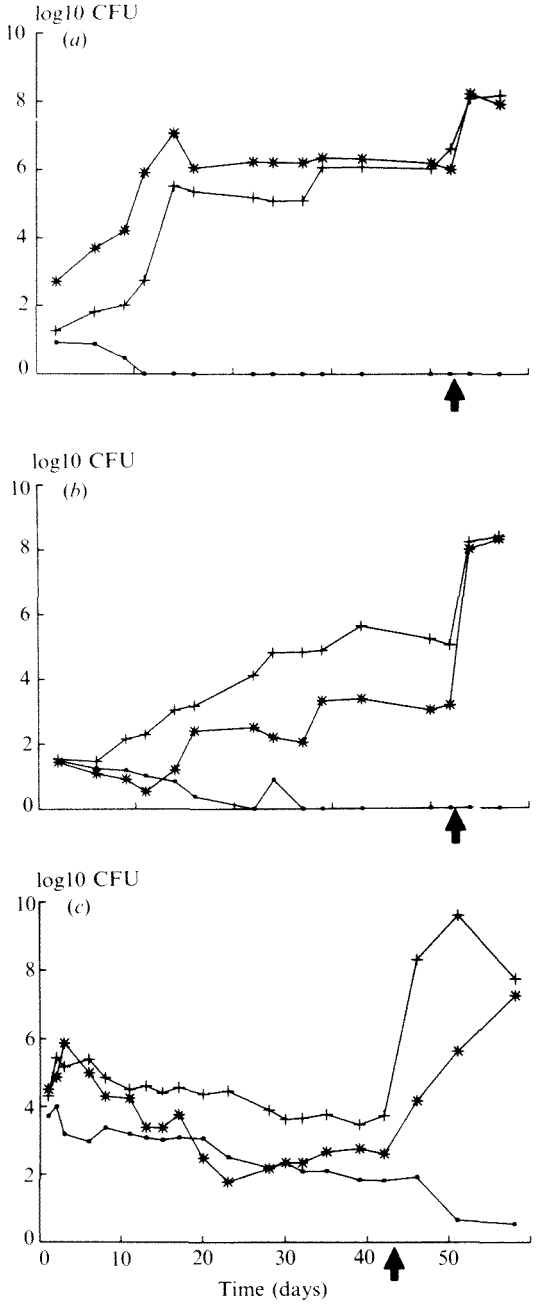

Fig. 1. The persistence of *S. enteritidis* (*a*) and (*c*), and *S. hadar* (*b*) at 4 °C, —●—; 20 °C, —+—; and 30 °C, —*—. Arrow indicates the addition of ferric ammonium citrate.

($> 1{\cdot}0 \times 10^7$) at 20 and 30 °C following the addition of ferric ammonium citrate to the albumen, e.g. Fig. 1c. It needs to be stressed that the generation times (< 12 h) in supplemented albumen was very short in contrast to those of *S. hadar*, for example, in unsupplemented albumen. The pH of the albumen changed (pH 9·5

https://doi.org/10.1017/S095026880004989X Published online by Cambridge University Press

Table 2. *The persistence of* Salmonella enteritidis *PT 4 in a piece of shell membrane in albumen* in vitro

	Persistence at			
	4 °C		25 °C	
Days	Membrane	Albumen	Membrane	Albumen
1	+	−	+	−
3	+	−	+	−
6	+	−	+	−
8	+	−	+	−
10	+	−	+	−
13	+	−	+	−
15	+	−	+	−
17	+	−	+	−

+, Organisms grew when membrane was placed on XLD and Nutrient Agar. −, No viable organisms grew from 0·1 ml of albumen placed on XLD and Nutrient Agar. The experiments were done in duplicate on two occasions.

to 7·0–8·0) when the populations of salmonellas attained $> 10^7$ cells/ml. Likewise the concentration of glucose in albumen diminished (50–70% loss) only when heavy growth occurred.

When *S. enteritidis* in the excised inner membrane of the air cell of eggs was suspended in albumen *in vitro*, it persisted in the membrane for upwards of 17 days at 4 and 25 °C. No viable organisms were recovered from the albumen (Table 2).

DISCUSSION

The studies by Clay and Board [16] demonstrated that, following inoculation of the inner shell membrane of the air cell with *S. enteritidis* PT4, there were two phases in the infection process of eggs stored at 10 or 25 °C. Persistence of the infection in the shell membrane together with contamination of albumen underlying the membrane with small numbers of quiescent organisms characterized the first phase. It was followed by rapid growth and gross infection of the albumen and yolk. Until recently there was general agreement based on circumstantial evidence coming from the many studies of infection of eggs with rot producing bacteria [18] that rapid multiplication occurs when organisms acquire essential nutrients on making contact with the surface of the yolk [19]. Humphrey and colleagues [4] have shown that salmonellas inoculated at the periphery of the albumen or at the mid point between the edge of the albumen and yolk in broken out eggs of 2–3 weeks of age failed to increase in numbers during 5 days of incubation at ambient temperature. In most instances those placed next to the yolk membrane remained in the lag phase for 2 days before increasing from 10^3 to 10^9 organisms per egg during the 2–4-day period of incubation. They deduced that storage changed the properties of the yolk membrane (vitelline membrane?) such that 'nutrients or some factors which negated the inhibitory properties of the albumen' accumulated around the yolk and 'reach(ed) a sufficiently high concentration to permit ... growth'. Future studies will need to address the changes occurring in the albumen of stored eggs and correlate these with the switch

from the quiescent to active growth phase of contaminating organisms. Further, such investigation could well aid interpretation of observations made in the present study.

We observed that non-proliferating salmonella inoculated into albumen remained motile for upwards of 42 days at 25 °C. The experiments with inoculated shell membranes suspended in albumen *in vitro* showed that the organism in the membranes remained viable but none was isolated from the albumen during 17 days incubation at 4 or 25 °C. These results, which are in accord with previous observations [20], lead us to suggest that organisms, particularly those in the infected shell membrane, move towards the food store of the yolk because of a gradient in the concentration of amino acids in the albumen. They multiply only when contact with the yolk provides other essential nutrients. Since the completion of this study we have obtained further evidence to support this hypothesis. These studies also suggested that the albumen's loss of viscosity, which occurs with storage, may facilitate microbial migration.

The present study has provided additional information on two subfeatures of the infection process immediately following trans-shell infection. Firstly, *Salmonella* serotypes and phage types exhibit a spectrum of response to egg albumen *in vitro*. It is noteworthy that among the organisms included in this study, *S. enteritidis* PT4 and *S. hadar*, both of which have been associated with infection of poultry and turkey flocks respectively [21], appear to be better adapted than the other serotypes in their capacity to remain viable or indeed grow feebly on some occasions in albumen at temperatures conducive to growth. Indeed, about a third of the *S. enteritidis* cells remained motile in unsupplemented albumen incubated at 25 °C for 42 days. The phenotypic attributes associated with this longevity and maintenance of motility are not known. It would be of interest to establish whether or not such attributes are also associated with those which cause *S. enteritidis* to be more invasive than other serotypes in young chicks [22].

Secondly, ferric ammonium citrate overcame the antimicrobial properties of the albumen for all 26 serotypes and phage types used in this study, providing incubation was at 20 or 30 °C. It has been shown repeatedly (see review by Tranter and Board [17]) that the bacteriostatic action of albumen against Gram-negative bacteria in general is negated by combined nitrogen and Fe^{3+} in amounts sufficient to saturate ovotransferrin. In such experiments the supplements were added before or immediately following inoculation of the albumen. In the present study bacteriostasis was released by supplements added on the 42nd day. It may be inferred, therefore, that the unsupplemented albumen induces a quiescent state in salmonella. This phenomenon was noted by Rozak, Grimes and Colwell [23] who worked on water contaminated naturally with this organism. This novel proposal may be linked with the observation [24] that the alkalinity (pH 9·6) of the albumen induces a marked change in the characteristics, e.g. increased heat resistance of these organisms.

ACKNOWLEDGEMENTS

This study was supported by a Ministry of Agriculture, Fisheries and Food studentship. One of us (J. L.) is grateful for their support. The support of the

following who supplied salmonella cultures is also gratefully acknowledged: T. Humphreys, Exeter PHLS; C. Wray, CVL (Weybridge); C. Baxter-Jones, *British United Turkeys*; *M. Hinton, Bristol University*.

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