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Salmonella enteritidis phage type 4 from the contents of intact eggs: a study involving naturally infected hens

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SUMMARY

Two small flocks of egg-laying hens, naturally infected with *Salmonella enteritidis*, were housed in individual cages so that their eggs could be identified. During a longitudinal study where the contents of 1119 eggs were examined, 11 were positive for *S. enteritidis*. One isolate was phage type (PT) 33 the others were PT4. The production of infected eggs was clustered though intermittent. The positive eggs, which were produced by 10 of the 35 hens, were all found to contain fewer than 10 salmonellas. Some birds were also apparently carrying *S. hadar* PT14 as this organism was isolated from the contents of six cracked eggs.

INTRODUCTION

In 1988 the Division of Enteric Pathogens (DEP) of the Public Health Laboratory Service (PHLS) received 15427 isolations of *Salmonella enteritidis* from human sources in England and Wales. Of these, 12522 (81·2%) were *S. enteritidis* phage type (PT) 4. Shell eggs are a major source of this organism (1).

The association between the contamination of eggs and human illness has led to attempts to quantify the risk to the public. Eggs tested in relation to cases or outbreaks can yield a significant proportion of positives (2) although eggs from incriminated flocks are often negative on subsequent re-testing (2). This observation suggests that the production of contaminated eggs may be intermittent and could possibly explain why routine, random testing rarely gives positive results (3).

It is important to establish both the prevalence of eggs contaminated with *S. enteritidis* PT4 and to estimate the numbers of this organism in the contents of naturally contaminated eggs. In an attempt to do this, birds from two naturally infected free-range flocks, one of which had been associated with a family outbreak of *S. enteritidis* PT4 infection (4) and the other a sporadic case (S. Mawer;

unpublished results), were housed in individual cages. This made it possible to identify the eggs laid by each bird. The contents of each egg was examined for the presence of salmonellas over a 3-month period.

This paper reports on the incidence of *S. enteritidis*-positive eggs laid by naturally infected hens. Information is also presented on the effects of shell quality and cleanliness on the presence of micro-organisms in egg contents.

MATERIALS AND METHODS

Hens

The birds used in this study comprised two naturally infected small flocks of free-range hens. One (flock Y), consisted of 23 hens. The other (flock P), comprised 15 birds, 12 of which were laying.

Following initial investigation by the PHLS Laboratory, Hull and the Veterinary Investigation Center, Lincoln the birds were taken to the PHLS Centre for Applied Microbiology and Research (CAMR). There, they were housed in individual cages in the animal house and fed a pelleted commercial feed and given water *as libitum*.

Cloacal swabs were taken from each bird on arrival at CAMR and sent to the PHLS Laboratory, Exeter for culture for salmonellas. These were examined using standard laboratory techniques.

Eggs

The hens were examined at least once per day and eggs were collected at each visit. Each egg was labelled, using a marker pen, with the identification code of the bird and the date of lay. The eggs were then stored, in clean egg boxes, at room temperature (c. 20 °C) until being sent to the Exeter laboratory for examination.

Microbiological Examination

On arrival at the Exeter laboratory the eggs were sorted into one of the following categories; (*a*) clean intact eggs; (*b*) intact eggs which were contaminated with faeces; (*c*) cracked eggs. They were then examined using the techniques described below.

Microbiological examination, in this study, was largely confined to detection and, where appropriate, enumeration of salmonellas in egg contents. Note was also made of the presence of contaminating organisms in the contents.

Shell decontamination

In this investigation it was clearly important to ensure that organisms present on the outer surface of the shell did not contaminate the egg contents. The following experiment was performed to determine optimum decontamination procedures.

Fresh faeces from free-range hens were inoculated with a diluted broth culture of *S. enteritidis* PT4 so that the number of salmonellas present was approximately $\log_{10}$ 6·0 per gram (wet weight). The actual inoculum size was estimated by performing a viable count on blood agar which was incubated at 37 °C for 24 h. Using a sterile wood tongue depressor at 1 cm^2 area on the shell of each of 60 fresh

intact battery eggs, from a flock regularly monitored and shown to be salmonella-free, was liberally inoculated with the faecal suspension. The eggs were placed in a 37 °C incubator for 4 h to allow the faecal material adhering to the shell to dry. On removal from the incubator, the contaminated area was ringed using a marker pen and the eggs separated, at random, into three groups of 20.

With one set, the faecal material was removed using a sterilized cotton wool pad moistened with sterile Ringer's solution. The rest of the shell was then cleaned using another similarly damp cotton wool pad. The eggs were either placed in 70% industrial methylated spirit (IMS) for three mins or wiped with a large swab impregnated with isopropyl-alcohol. With the second set, the faeces were not removed, the eggs being placed directly into IMS or wiped with the alcohol-impregnated swabs. The last group was not subjected to either of the above procedures. With either method of disinfection the alcohol was allowed to evaporate and the egg contents were removed for culture by breaking the shell, at the site of contamination, against the rim of a sterile 250 ml screw-capped jar. The egg contents were cultured for salmonellas as described below.

Salmonella culture

Shell decontamination was largely as described above with the following exceptions; as an additional precaution against cross-contamination, the eggs were broken by cracking a previously clean area of the shell against the rim of the jar. The member of staff carrying out the examination also wore a separate pair of clean disposable gloves for each egg. In the early part of the investigation at the Hull Public Health Laboratory the swabs used to clean the shells prior to alcohol disinfection were impregnated with Buffered Peptone Water (BPW) and were cultured for salmonellas.

The contents of intact eggs were either homogenized by shaking vigorously for 30 s or the yolk and white were separated and cultured separately. With each cracked egg the yolk and white were cultured separately. Ten ml of either the homogenized contents or the yolk or 5 ml of the white were transferred, using a sterile wide-bore 10 ml pipette to a sterile 25 ml screw-capped bottle and stored at 4 °C. Approximately 200 ml of BPW was then added to each of the jars which were incubated at 37 °C for 18-24 h. Following incubation, 0·2 ml BPW was inoculated into 20 ml Rappaport Vassiliadis broth (RV; Oxoid) which was incubated at 41 °C for 24 h. As well as inoculation of RV broth, each pre-enrichment broth was also streaked directly onto Xylose Lysine Deoxycholate agar (XLD; Oxoid). If these plates indicated that the BPW cultures contained a heavy population of contaminating organisms, which might have masked the growth of salmonellas, the appropriate RV broths were also streaked onto XLD.

Where enrichment culture demonstrated the presence of salmonellas in the egg contents the most probable number (MPN) of salmonellas present in the retained portion was estimated by carrying out the following; 5 ml was added to 20 ml of BPW and 5×1 ml to 5×10 ml BPW. The inoculated mixtures were then cultured for salmonellas as described previously.

All salmonella-like colonies were confirmed by biochemical and serological testing and were sent to the PHLS Division of Enteric Pathogens (DEP) for confirmation and phage typing.

Table 1. *The influence of shell decontamination on the cross-contamination of egg contents during the breaking of eggs*

Group	Decontamination procedure	Number of eggs with salmonella-positive contents*
A	None	17
B	Immersion in IMS for 3 min	17
C	Removal of faeces followed by immersion in IMS for 3 min	0†

* In each experimental group 20 eggs were contaminated with fresh hens' faeces inoculated with *S. enteritidis* PT4. The level of contamination per egg was approximately $\log_{10}$ 5·8 salmonellas.

† Only one BPW pre-enrichment culture contained coliforms.

Contamination of egg contents with organisms other than salmonellas

To examine the influence of shell surface contamination and shell integrity on the contamination of egg contents with micro-organisms a note was made of the presence of organisms in the pre-enrichment BPW broths as demonstrated by growth on the XLD plates. These data were then correlated with those on the condition of the egg shell.

Statistical analysis

The differences in the effectiveness of the decontamination procedures and in the effects of the condition of the shell surface on internal contamination were compared using chi-square tests. The distribution of eggs with contents positive for *S. enteritidis* examined using the Poisson Heterogeneity test.

RESULTS

Shell decontamination

Removal of adhering faecal material and cleansing of the shell surface followed by disinfection in IMS or with a swab impregnated with isopropyl alcohol successfully prevented the cross contamination of the egg contents during their removal from the shell (Table 1). This technique which was adopted for the bulk of the study was shown to be suitable even though the level of faecal contamination on the shells of the eggs used in the experiment greatly exceeded that found on commercially produced eggs and on those examined in the study. The results for the two methods of shell disinfection were essentially the same and for clarity those for immersion in IMS only are shown in Table 1.

The incidence of eggs with salmonella-positive contents

Four hundred and fifty-one eggs from flock P and 68 flock Y were examined before the birds were placed in individual cages. *Salmonella enteritidis* PT4 was isolated from both shells and contents (Table 2). Cloacal swabs taken on arrival at CAMR were all negative for salmonellas.

The hens were caged on 27 January 1989. From then until 17 April 1989 the

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Table 2. *The isolation of* Salmonella enteritidis* *from the shells and contents of eggs laid by two naturally infected flocks*†

Flock	No. of eggs examined	No. of eggs salmonella-positive	%	Site(s) of contamination
P	451	5‡	1·1	3 yolk only 1 albumen only 1 homogenized contents
Y	68	7	10·3	1 yolk only 1 albumen only 5 shell only

* One isolate of *S. enteritidis* from an egg shell was not phage typed. The rest were *S. enteritidis* PT4.

† Birds were not housed individually.

‡ All shells from flock P were salmonella-negative.

Table 3. *The incidence of* Salmonella enteritidis-*positive contents in eggs laid by two naturally infected flocks**

	Flock P				*Flock Y*		
Bird No.	No. of eggs laid	No. of positive eggs	%	Bird No.	No. of eggs laid	No. of positive eggs	%
1	70	0	—	26	4†	0	—
2	68	1	1·5	31	14†	0	—
3	58	0	—	32	5†	0	—
5	56	1	1·8	33	8†	0	—
6	28	0	—	34	31	2	6·5
7	60	1	1·7	35	34†	0	—
8	22	0	—	36	32†	0	—
9	60	1	1·7	37	34†	0	—
11	54	1	—	39	15†	0	—
12	67	1	1·5	41	7†	0	—
15	57	0	—	42	39†	0	—
711	67	1	1·5	44	21†	0	—
				45	21†	0	—
				46	8†	0	—
				47	24†	0	—
				48	24†	1	4·2
				49	14†	0	—
				50	27†	0	—
				52	10†	0	—
				55	50	1	2·0
				57	30†	0	—
Total	667	7	1·1		452	4	0·9

* The birds were housed individually.

† Birds killed on 7 April 1989.

homogenized contents of 1119 eggs were examined. Seven (1·1 %) of 667 from flock P were positive for *S. enteritidis*. One isolate was PT33 the others were PT4. Four (0·9 %) of 452 eggs from flock Y were positive for PT4 (Table 3). In all cases the organism was isolated, in pure culture, from the pre-enrichment broths. For the whole period the birds were under investigation, December 1988–April 1989, 18 (1·1 %) of 1638 eggs contents were positive for *S. enteritidis*.

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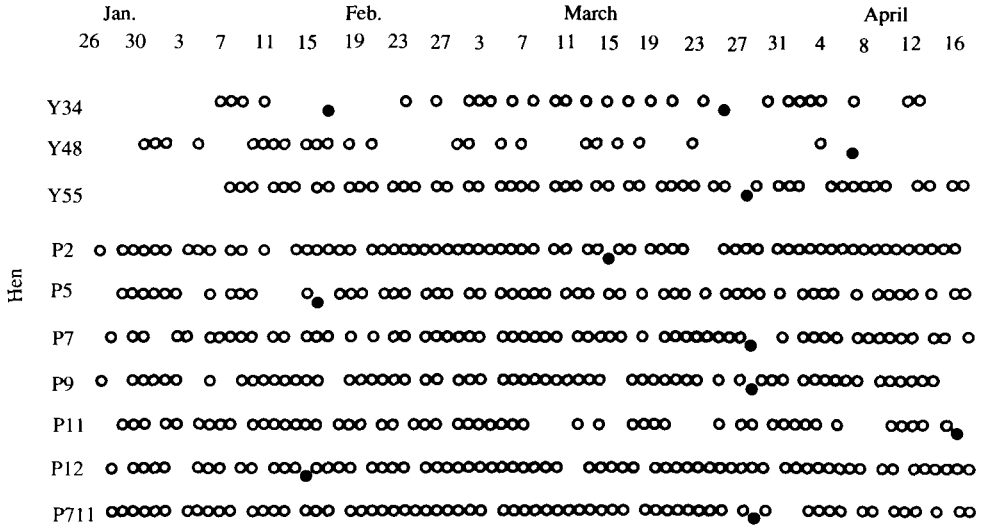

Fig. 1. The distribution of egg contents positive for *Salmonella enteritidis*. ●, Egg contents positive for *S. enteritidis*; ○, negative eggs. With the exception of the isolate from the egg laid by hen P5 on 16 February 1989, which was PT33, all the organisms were *S. enteritidis* PT4.

Table 4. *The variation in the incidence of egg contents positive for* Salmonella enteritidis *on days when at least one egg was positive*

Date	No. of eggs laid*	No. of eggs salmonella-positive*	%
1989			
15 Feb.	18	1	5·6
16 Feb.	24	1	4·1
17 Feb.	16	1	6·3
15 Mar.	12	1	8·5
26 Mar.	11	1	9·9
28 Mar.	18	4	22·2
7 Apr.	14	1	7·1
16 Apr.	9	1	11·1

* The information from the two flocks has been combined.

In 10 of the eggs examined in the latter part of the study the shell was intact. In the other egg it was cracked although the organism was present only in the yolk. The number of salmonellas in the retained portion of all the intact eggs was less than 10/100 ml.

When it became possibly to identify each bird it was found 10 of the 35 birds in the study produced eggs positive for *S. enteriditis* (Fig. 1; Table 3) with one bird, Y34, laying two contaminated eggs. Seven of the birds were from flock P and three from flock Y (Table 3). With the exception of birds Y34 and Y48 which laid fewer eggs overall, the incidence of positive eggs per bird was remarkably constant at approximately 1·5–2 % (Table 3).

The production of contaminated eggs was intermittent and between the positive eggs on 16 February and 15 March a total of 393 were examined and found to be

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Table 5. *The influence of shell condition on the contamination of egg contents with micro-organisms*

Group	Shell condition	No. of eggs	No. of eggs salmonella-positive	%	No. of eggs with contaminated contents*	%
A	Clean and intact	665	6†	0·9	74	11·1
B	Intact and contaminated with faeces	290	4†	1·4	118	40·7
C	Cracked	164	7‡	4·3	115	70·1

* Principally *Escherichia coli*.

† *Salmonella enteritidis*.

‡ Six isolates *Salmonella hadar*; 1 isolate *S. enteritidis* (from yolk only).

Groups A and B did not differ in the incidence of salmonella-positive contents ($\chi^2 = 0{\cdot}48$; $P > 0{\cdot}99$). Cracked eggs were significantly more likely, however, to contain salmonellas ($\chi^2 = 9{\cdot}7$; $P < 0{\cdot}01$).

negative. There would also appear to be a tendency for salmonella-positive eggs to be laid at the same time by different birds. Thus three eggs were produced in the period 15–17 February and five between 26–28 March (Fig. 1). This apparent clustering, which was not supported by statistical analysis, meant that on days when contaminated eggs were laid their incidence greatly exceeded the overall figures quoted in Table 2 and ranged from 4·1 to 22·2% (Table 4). The contents of six other cracked eggs were positive for *S. hadar* PT14. No attempt was made to enumerate these organisms.

The influence of the condition of the shell on the contamination of egg contents with salmonellas and coliforms.

Cracked eggs were more likely to contain salmonellas than those with intact shells even if the shells of the intact eggs were contaminated with faeces (Table 5). The distribution of salmonellas differed between the two groups. *Salmonella enteritidis* only was isolated from the contents of intact eggs whereas in cracked eggs *S. hadar* predominated (Table 5).

Eggs with intact, clean shells were much less likely to contain coliforms than those with either dirty or cracked shells (Table 5). In the latter group the contents of 70% of the eggs were contaminated (Table 5).

DISCUSSION

The results presented in this paper are in agreement with those in other recent reports (2, 4–6) and demonstrate, once again, that it is possible to isolate *S. enteritidis PT4* from the contents of intact shell eggs. This study had attempted to go further than just demonstrating the presence or absence of salmonellas in egg contents. It is the first both to identify individual eggs with the naturally infected birds that laid them and to attempt to count the number of salmonellas in contaminated egg contents. No attempt was made, in the latter part of the study, to examine shells for the presence of salmonellas even though this can be rewarding and can help to identify positive flocks (2, 7).

The examination of eggs from flocks implicated in cases or outbreaks of *S. enteritidis* PT4 infection (2) suggested that the production of contaminated eggs may be intermittent. The distribution of contaminated eggs in Fig. 1 lends support to that suggestion although statistical analysis failed to reveal any significant associations. It is possible, however, that some stimulus is causing different hens to lay contaminated eggs at or around the same time (Fig. 1; Table 4). Such a pattern of distribution of contaminated eggs means that routine, non-targeted, sampling of eggs is unlikely to be productive and could lead to a false sense of security. The results presented in Fig. 1 and Table 4 show, however, that on some days a significant proportion of eggs may have *S. enteritidis* PT4 in their contents.

The isolation of *S. enteritidis* PT4 from the yolks of intact eggs and from those of soft shell eggs and ovules taken at post-mortem (8, 9) indicate that this organism can be transmitted vertically. In this respect it differs from *S. thompson* (10), *S. anatum* (11) but behaves in a manner similar to *S. pullorum* (10) and *S. menston* (12).

A number of salmonellas, including *S. enteritidis* PT4, are able to grow rapidly at room temperature in artificially contaminated egg yolks or homogenized whole egg (13, 14). This would not appear always to be the case with naturally contaminated eggs. Those identified as being positive in this study had sometimes been stored at ambient temperature (20 °C) for 5 days before examination. Despite this, all were found to contain fewer than 10 salmonellas per egg. This level of contamination is similar to that previously reported for homogenized whole egg before pasteurization (15). As has been suggested before (2, 4) the low numbers of organisms in an egg could be due to either micro-colonies within the yolk or death of the salmonellas during storage. The former is unlikely to be a relevant factor here as the egg contents were fully homogenized before examination. Initial studies (unpublished results) have also shown that the number of salmonellas does not change significantly in either egg yolk or homogenized whole egg during storage at 4 °C for 48 h. There would appear to be, therefore, additional factors which control the growth of salmonellas in naturally contaminated eggs. One of these could be the presence of antibody against *S. enteritidis* PT4 in the yolk (16).

In the United Kingdom advice has been issued (17) concerning the potential dangers of using cracked and/or faecally contaminated eggs and this paper provides evidence to support this view. Eggs with shells that were contaminated with faeces or that were cracked were significantly more likely to contain coliforms (Table 5). More importantly, cracked eggs were more frequently contaminated with salmonellas (Table 5). It is interesting to note that intact eggs contained *S. enteritidis* only whereas, in the main, cracked eggs were contaminated with *S. hadar* PT14. The former organism was presumably present as the result of trans-ovarian transmission while the latter may have penetrated the damaged shell (18) as a result of faecal contamination.

The invasive nature of *S. enteritidis* PT4 (19) can lead to its presence in egg contents. This permits the organism to survive a variety of forms of cooking common in domestic and commercial kitchens (11) and represents a significant potential threat to public health. For that reason this investigation has concentrated upon that aspect of egg contamination. This organism can also be isolated, however, from egg shells (Table 2). It was important, therefore, in this

study to use a technique that would prevent the contamination of egg contents during their removal from the shell. The PHLS technique of removal of adhering faecal material by swabbing followed by immersion of the egg in IMS has been shown (Table 1) to be successful in preventing cross-contamination even when the eggs were subjected to gross faecal contamination.

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