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Numbers of *Salmonella enteritidis* in the contents of naturally contaminated hens' eggs

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SUMMARY

Over 5700 hens eggs from 15 flocks naturally infected with *Salmonella enteritidis* were examined individually for the presence of the organism in either egg contents or on shells. Thirty-two eggs (0·6%) were positive in the contents. In the majority, levels of contamination were low. Three eggs, however, were found to contain many thousands of cells. In eggs where it was possible to identify the site of contamination, the albumen was more frequently positive than the yolk. Storage at room temperature had no significant effect on the prevalence of salmonella-positive eggs but those held for more than 21 days were more likely ($P < 0·01$) to be heavily contaminated. In batches of eggs where both shells and contents were examined, 1·1% were positive on the former site and 0·9% in the latter.

INTRODUCTION

Salmonella enteritidis phage type (PT)4 can be isolated from the contents of clean, intact shell eggs [1–4]. Estimates of the prevalence of salmonella-positive eggs varies. A PHLS survey in 1988–9, of eggs from sources though to be implicated in cases or outbreaks of *S. enteritidis* infection, found that approximately 0·1% of contents were positive [5]. Investigation with individual flocks, however, has demonstrated that on certain occasions, *S. enteritidis* can be isolated from the contents of up to 50% of the eggs examined [1]. These, and other results [3, 4] suggested that there was clustering of salmonella-positive eggs and that the chances of detecting contamination was influenced by the timing of sampling in addition to the techniques used.

In common with other salmonellas, *S. enteritidis* PT4 can grow rapidly in the yolks [6] and, under some conditions, the albumen [7] of artificially contaminated eggs stored at room temperature. When present in the yolk, the organism has been shown to survive a variety of forms of cooking [6]. In can also be isolated (Humphrey, unpublished results) from the albumen of eggs cooked under simulated domestic conditions. Analysis of cases and/or outbreaks of *S. enteritidis* infection [8, 9] have revealed a significant association between the consumption of scrambled egg, lightly cooked eggs, shop bought egg sandwiches and illness. The

demonstration that dishes such as scrambled egg have caused *S. enteritidis* infection implies that some naturally contaminated eggs must have contained large numbers of cells before cooking [6].

In one of the few studies where an attempt was made to enumerate the number of salmonellas in the contents of naturally contaminated eggs, all those examined, using a most probable number technique, were found to contain less than 10 cells per egg despite being stored at room temperature for up to 7 days [4]. This study was based on two small flocks housed under experimental conditions and may not have been representative of commercial conditions. To gain a better understanding of the prevalence of salmonella-positive eggs, the number of cells in egg contents, their position and the influence of storage at room temperature on levels of contamination, eggs were obtained from naturally infected, commercial egg-laying flocks at or around the time of either associated human cases of *S. enteritidis* PT4 infection or when routine monitoring by the Ministry of Agriculture, Fisheries and Foods (MAFF) revealed flock infection. Eggs were examined for the presence of salmonellas on the shells and in either separated or whole homogenized egg contents. The majority of eggs were examined between March and October 1990 although some information is given on eggs examined between June 1988 and February 1989. The results of these investigations are presented along with details of the effects of a variety of parameters on the growth of *S. enteritidis* PT4 in artificially contaminated yolks and albumen.

MATERIALS AND METHODS

Eggs

Eggs were either purchased from flocks identified by MAFF as infected with *S. enteritidis* or examined as part of investigations of either human cases or outbreaks. The eggs were stored at ambient temperature (*c.* 21 °C) for up to 35 days before microbiological examination.

Eggs were inspected before examination and those that were either cracked or showed heavy faecal contamination were either discarded or examined separately.

Microbiological examination

Egg contents and, on occasion, shells were examined for the presence and numbers of salmonellas using previously published techniques [4]. To ensure that there was no cross-contamination from other laboratory activities, eggs were stored in a clean room and were removed to a separate room, dedicated to the purpose, for testing. Examination of plates and identification of salmonellas was carried out in a third room.

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

Artificially contaminated eggs

Cultures of *S. enteritidis* PT4 previously isolated from eggs were obtained from DEP and were used to contaminate, in a manner previously described [6], the contents of intact shell eggs, obtained from either flocks shown to be uninfected

with *S. enteritidis* or naturally infected hens found to have high levels of circulating antibodies against *S. enteritidis* [10]. The level of the inoculum and its position within the egg were varied so that the effects of these parameters on the growth of *S. enteritidis* in intact eggs could be studied.

Eggs which were either less than 7 days or more than 2 weeks old were also broken, using aseptic techniques, into petri dishes. The albumen was inoculated with between 10^2 and 10^5 cells of *S. enteritidis* so that the organism was either next to the yolk membrane, at the outer edge of the albumen or equidistant between these two points. The inoculated eggs were stored in the dark at ambient temperature for up to 7 days before microbiological examination. With some eggs, the yolk and albumen were separated and placed in sterile 25 ml screw-capped bottles. These were inoculated with either 10, 10^2, 10^3 or 10^4 cells of *S. enteritidis* and stored as above.

Statistical analysis

The significance of the differences in the distribution of salmonella-positive eggs between the various groups of egg-laying hens and between eggs stored for either more or less than 21 days was examined using χ^2 tests.

RESULTS

The prevalence of salmonella-positive egg contents

Salmonella enteritidis was isolated from the contents of 32 eggs (0·6%). One isolate, from an egg laid by a layer-breeder hen, was *S. enteritidis* PT4a. All other isolates were PT4. No other type of salmonella was found in egg contents.

The prevalence of contamination of the contents of the eggs from the layer-breeder flock was significantly lower ($P < 0{\cdot}01$) than that of the eggs from the other flocks (Table 1).

The influence of storage at ambient temperature on the numbers of salmonellas in egg contents

Eggs which were examined as part of an investigation of either a case or an outbreak of *S. enteritidis* infection, were tested within 24 h of arrival at the laboratory. Their age ranged from 48 h to 4 weeks post-lay. One egg, which had been held at ambient temperature in the kitchen of a restaurant for 3 weeks before microbiological examination, was shown to be salmonella-positive by culture of a loopful of homogenized contents on an XLD plate. This indicates that many cells of *S. enteritidis* were present. All other salmonella-positive eggs examined during the investigation of human infection and where enumeration was attempted, were found to contain less than 10 salmonellas. Most of these eggs were less than 7 days old at testing.

Eggs from flocks identified by MAFF were received between 1 and 14 days after lay. They were stored at ambient temperature and examined, at regular intervals, in lots of either 100 or 200.

Storage had no effect on the prevalence of salmonella-positive eggs (Table 2) but appeared to influence levels of contamination. Thirteen (0·4%) of 3659 eggs stored

Table 1. *The influence of either bird or flock type on the prevalence of the contamination of egg contents with* Salmonella enteritidis

Flock type	No. of flocks	No. of eggs examined	No. with salmonella-positive contents	%
Free-range	6	1883	12	0·64
Battery	6	1229	9	0·73
Layer-breeder	1	1120	1	0·09
Broiler-breeder	2	1558	10	0·64
Total		5790	32	0·55

Table 2. *The influence of storage at room temperature on the frequency of isolation of* Salmonella enteritidis *from the contents of naturally contaminated eggs and on the numbers of cells present*

Days at room temperature	No. of eggs examined	No. positive for *S. enteritidis*	(%)	No. of eggs containing salmonellas at the following levels/egg			
				< 20	< 100	> 100	> 1000
0–7	1085	5	(0·5)	5	0	0	0
8–14	1353	7	(0·5)	7	0	0	0
15–21	1221	1	(0·1)	1	0	0	0
> 21	1603	12	(0·8)	7	0	3	2*

* One egg contained $1{\cdot}5 \times 10^4$ cells of *S. enteritidis* and the other contained $1{\cdot}2 \times 10^5$.

for up to 21 days were salmonella-positive. None contained more than 20 cells of *S. enteritidis* This was in marked contrast to the levels of contamination in eggs stored for more than 3 weeks (Table 2). Twelve (0·5%) of 1603 were salmonella-positive with five containing more than 100 cells ($P < 0{\cdot}01$). Two eggs, each stored for 21 days, contained $1{\cdot}5 \times 10^4$ and $1{\cdot}2 \times 10^5$ cells of *S. enteritidis* PT4 respectively.

As a general rule, the majority of the contents of clean, intact shell eggs contained few salmonellas, irrespective of the length of storage at room temperature. Thus, 23 (72%) of the overall total of 32 salmonella-positive eggs contained less than 20 cells of *S. enteritidis*.

The relative frequencies of the contamination of either egg shells or contents with salmonellas

The contents of each egg examined during outbreak investigation was cultured separately whereas the shells were often tested in bulk. *Salmonella enteritidis* PT4, *S. typhimurium* DTs 49, 110 and 141 and *S. hadar* PT14 were isolated from egg shells. The contents yielded only *S. enteritidis*.

With some batches of eggs, each shell and contents of each egg were identified and cultured separately. From 1952 eggs examined in this manner, *S. enteritidis* was isolated from the shell of 21 (1·1%) and the contents of 18 (0·9%). Only one egg was positive in both sites.

It was also possible, with 15 salmonella-positive eggs, to identify the site of contamination within the egg. *Salmonella enteritidis* PT4 was isolated from the

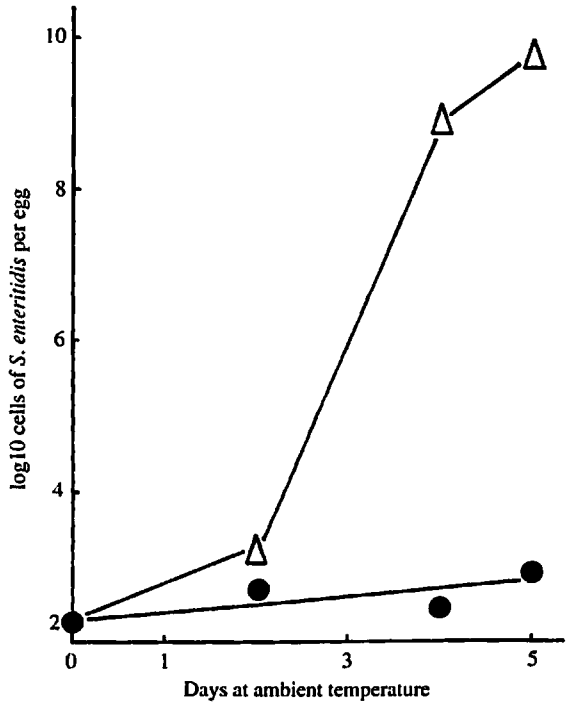

Fig. 1. The influence of position within the albumen on the growth of *S. enteritidis*. Eggs were more than 2 weeks old on inoculation. △, Organism placed next to the yolk membrane; ●, organism placed at either the outer edge of the albumen or at a point equidistant between that site and the yolk membrane.

albumen of 12 (80%), from the yolk of two eggs (13%) and from both sites in one egg. In these examinations the yolk, its membrane and some adhering albumen were cultured together.

The growth of Salmonella enteritidis *PT4 in artificially contaminated egg contents*

The level of the inoculum, in the range 5–100 cells per egg, had no effect on the growth of *S. enteritidis* PT4 in the artificially contaminated yolks of intact shell eggs, with the organism growing rapidly in eggs stored above 10 °C. The bacterium also grew equally well in the yolks of eggs laid by either infected hens with high levels of circulating antibodies against *S. enteritidis* or birds showing no evidence of infection. *Salmonella enteritidis* was also capable of good growth on a medium comprising only agar and egg yolk whether the plates were incubated either aerobically or anaerobically.

The growth of *S. enteritidis* in albumen was governed by the age of the egg on inoculation and the position of the organism in relation to the yolk membrane.

In eggs that were more than 2 weeks old there was a rapid increase in the number of cells of *S. enteritidis*, from an initial inoculum of 10^2 cells, when the organism was placed next to the yolk membrane (Fig. 1). Little growth occurred when the inoculum was placed elsewhere in the albumen (Fig. 1) or in separated albumen from the same batch of eggs (results not shown).

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With fresh eggs, *S. enteritidis* did not grow in any position in the albumen if the inoculum was less than 10^3 cells per egg. When it exceeded this level, growth took place in a minority of eggs (3/20) although this was largely unrelated to the position of the organism within the egg or the size of the inoculum. Thus, in some eggs growth did not take place even when $1{\cdot}5 \times 10^4$ cells were placed next to the yolk membrane.

In eggs where *S. enteritidis* did not grow in the albumen, the numbers of cells did not alter significantly during the storage period. Survival was unaffected by either the storage temperature, in the range 10–25 °C, or the size of the inoculum, in the range 10–10^5 cells.

DISCUSSION

Since 1987–8 contaminated, intact shell eggs have been shown to be an important vehicle for human infections with *S. enteritidis* [5]. There is strong circumstantial evidence that some naturally contaminated eggs must have contained large numbers of cells. This came principally from the observation that either cases or outbreaks of *S. enteritidis* infection had been caused by cooked dishes such as scrambled egg [8, 9]. Research into the survival of salmonellas during the stimulated domestic cooking of eggs [6] had demonstrated that for viable cells to be isolated from scrambled egg a large initial inoculum was necessary.

The indirect evidence was not supported by reports on the examination of naturally contaminated eggs which demonstrated that all contained less than 20 cells even though some had been stored at ambient temperature for up to 7 days before examination [2–4]. The results presented in this paper demonstrate unequivocally, however, that naturally contaminated clean, intact shell eggs can contain large numbers of *S. enteritidis*. Two eggs, from a small free-range flock, were found to contain $1{\cdot}5 \times 10^4$ and $1{\cdot}2 \times 10^5$ cells of *S. enteritidis* respectively. The organism was present in pure culture, as it was in 31 (97%) of the 32 salmonella-positive eggs. The site of contamination was not identified in the two heavily contaminated eggs but results presented in this paper strongly suggest that it was likely to be the albumen.

All the eggs that were heavily contaminated (> 100 cells per egg) were more than 2 weeks old (Table 2). In a small survey of retail outlets in Plymouth (Altari, personal communication) the mean shelf life of eggs was found to be $17 \pm 1{\cdot}0$ day with the range being between 10 and 25 days.

Salmonella enteritidis PT4 can be differentiated from most other poultry-associated salmonellas that are non-host-adapted in that it can be transmitted vertically from infected parent birds and can thus be isolated from reproductive tissue and the contents of intact ova [1, 12]. The latter isolations in particular, may have, understandably, led to the belief that the principal site of contamination within shells eggs is the yolk. This seems not to be the case. In this investigation, there is both direct and indirect evidence that the albumen, or possibly the yolk membrane [13], is more likely than the yolk to be contaminated with *S. enteritidis*. The organism is capable of achieving large populations in yolk contents from any inoculum, at a range of temperatures from 10 to 45 °C and under either aerobic or anaerobic conditions, whether the yolk came from eggs laid

by hens with high levels of circulating antibodies, where presumably the yolk contained large concentration of antibodies [14], or hens showing no signs of infection. Thus, if yolk contents were frequently contaminated it would not be unreasonable to expect that the majority of eggs would contain large numbers of cells. This is not the case and 72% of the eggs found positive in this study contained less than 20 cells of *S. enteritidis*. This strongly suggests that the albumen is the principal site of contamination and the organism was isolated from this material in 12 of 15 eggs where the yolk and albumen were cultured separately. This is in agreement with recently published work on egg-laying hens artificially infected with *S. enteritidis* [15] where, although the organisms was isolated, with high frequency, from albumen, all yolk contents were salmonella-negative. It should be remembered that the isolation of *S. enteritidis* from ovarian tissue [1, 12] indicates that yolk contamination, albeit at a relatively low frequency, is a possibility and this can lead to eggs carrying very high populations of the organism.

The results with naturally and artificially contaminated eggs appear to demonstrate that during the passage of the egg through the oviduct the albumen is seeded with a few cells of *S. enteritidis*. These remain dormant, even in eggs stored at ambient temperature, for 2–3 weeks. During that time physical and chemical changes take place in the egg contents [16]. A consequence of these would seem to be that either nutrients or some factors which negate the inhibitory properties of the albumen leak out from the yolk, possibly because of alterations in the structure of the yolk membrane (Gane 1935, quoted in [16]). In the area of the albumen closest to the yolk the growth promoting factors reach a sufficiently high concentration to permit the growth of *S. enteritidis* (Fig. 1) and to support large populations of this organism.

This view is supported by the elegant experiments of Board and his co-workers [7] who observed that migration of the yolk towards air sacs contaminated with *S. enteritidis* enhanced multiplication of the organism. Changes in the albumen alone are not sufficient to permit growth as, in the majority of eggs, the organism grew neither when inoculated into sites away from the yolk nor in albumen stored for 7 days at ambient temperature.

Delays in the growth of *S. enteritidis* in albumen have also been seen with other salmonellas in artificially contaminated eggs held at ambient temperature [17, 18]. Length of storage of naturally contaminated intact eggs had no effect, however, on the numbers of salmonellas in homogenized egg prior to pasteurization [19]. This was presumably because as the study was carried out in 1968 the eggs would have been contaminated on the shell with salmonellas other than *S. enteritidis* and the numbers of organisms would have declined during storage.

In the investigations reported in this paper, together with earlier studies [2, 4] a total of 6909 eggs from flocks naturally infected with *S. enteritidis* have been examined for the presence of salmonellas in their contents. Forty-three (0.6%) were positive for *S. enteritidis*. In three eggs (0·04%) many thousands of salmonellas were present. This level of contamination poses a potential threat to public health if the eggs were not cooked properly and demonstrates that the advice on the cooking and handling of eggs issued by the Chief Medical Officer of the United Kingdom in 1988 [20] is still relevant in 1990.

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