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Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens

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SUMMARY

Two Enteritidis PT4 isolates which differed in inherent tolerance to heat, acid, H_2O_2 and the ability to survive on surfaces were used to infect mice, day-old chicks or laying hens. The acid-, heat-, H_2O_2- and surface-tolerant isolate was more virulent in mice and more invasive in laying hens, particularly in reproductive tissue. However, no significant differences were observed in behaviour in chicks. Both PT4 isolates were able to infect chicks housed in the same room as infected birds, although the heat-tolerant isolate survived significantly better than the heat-sensitive one in aerosols.

INTRODUCTION

Salmonella enterica serotype Enteritidis continues to be an internationally important human pathogen and predominates in human salmonellosis in countries in both the northern and southern hemispheres. Enteritidis comprises over 50 phage types (PT), although PT4 would appear to be the most widespread. Outbreaks with this phage type have been associated with the consumption of cooked and acidified foods [1]. As part of a continuing study into the pathogenicity of PT4 the PHLS Food Microbiology Research Unit screened 40 isolates of PT4, obtained from human cases, chicken or eggs, for differences in tolerance to a range of stressful conditions [2]. Results revealed marked differences between isolates [2] and demonstrated that strains which were heat-tolerant were also more acid-tolerant and survived significantly better in the presence of hydrogen peroxide (H_2O_2) and on surfaces. Conversely, isolates which were heat-sensitive were also acid-, H_2O_2- and surface-sensitive [2]. As cultures were grown under standard, non-inducing conditions at pH 7.0 and 37 °C the observed tolerances were termed 'inherent'.

Previous work in other laboratories demonstrated that naturally occurring variants of Enteritidis PT13a differed in their ability to contaminate egg contents, invade spleens of 5-day-old chicks and in the quantity of 0-antigen neutral sugar present [3, 4]. However, a study in which day-old chicks were infected with four different strains of PT4 showed no significant differences in tissue invasion or mortality [5]. Later work by the same group [6], where PT4 isolates from either chickens or humans in 1982 were compared with similar isolates from 1988, suggested that the later strains might be more invasive in chicks [6]. These workers also conducted experiments which indicated that isolates of PT4 might be more invasive in chicks than Enteritidis PTs 7, 8 or 13a [7]. Investigations using a few strains of mice found that of a number of Enteritidis PTs examined only PT4 produced a lethal effect in BALB/C mice [8]. There were differences in

effects between mouse strains which have also been noticed by other researchers [9].

The above investigations suggest that there may be variation in the virulence of Enteritidis isolates both between and within phage types. There has, however, been little work to examine links between tolerance to adverse environments and observed pathogenicity. Given the continued importance of Enteritidis PT4 as a human pathogen it was decided to examine the behaviour, in mice and chickens, of two of the PT4 isolates previously subjected to detailed analysis of tolerance and survival profiles [2]. As PT4 is also able to infect chickens either by aerosols [10] or via the conjunctiva [11], the survival of the isolates in air was also studied.

MATERIALS AND METHODS

Bacteria

Two isolates of Enteritidis PT4 were used. Both were isolated in 1993. Isolate E, which has enhanced 'inherent' tolerance to heat, acid and H_2O_2 and survives significantly longer on surfaces [2], was obtained from a human case. Isolate I, which is heat-, acid-, H_2O_2- and surface-sensitive [2], came from a fresh chicken carcase purchased from a local retail outlet. The letters E and I are used for identification purposes only and to enable correlation with previous data published on these isolates [2].

Strains were maintained on blood agar incubated at 37 °C. To obtain inocula for animal experiments, cultures were standardized as previously described [2] and incubated for either 3 or 15 h to obtain either log or stationary phase cells respectively.

Animal studies

Mice

Three experiments were conducted using the C57/BL6/J mouse strain at 20 g weight (*c.* 4 weeks of age). The animals were infected into the oesophagus with *c.* 10^7 cells of PT4, strain E or I, in 0·1 ml of culture broth. The mice were caged individually and fed on a non-irradiated salmonella-free commercial diet (Bond K Universal, Hull). Food and water was supplied *ad libitum*. The mice were observed at least daily for signs of illness such as ruffled fur, hunched backs, white rings around the eyes and lethargy. Those showing all these symptoms were considered likely to die and were killed by carbon dioxide asphyxiation. Experiments were terminated after 2 weeks when all mice remaining alive were killed as above. Samples of liver, spleen and blood were taken using aseptic techniques and cultured for salmonellae using standard pre-enrichment, enrichment and plating techniques [10, 11]. In two experiments, 15 h (stationary phase) cultures were used. In the other, mice were infected with log phase (3 h) cultures.

Chicks

Two experiments were performed using day-old chicks. Fertile hatching eggs from a salmonella-free commercial breeder flock of Hisex Brown birds were hatched in an incubator. Chicks were divided, at random, into two groups of 20 birds. They were infected by direct intubation into the crop with *c.* 10^7 cells of PT4 isolate E or I. The two groups of infected birds were placed in separate rooms and were kept in an open run and provided salmonella-free feed and water *ad libitum*. In one experiment, ten control chicks were kept in a pen next to each group of infected birds.

Birds were observed at least once per day and any chicks found to be dead were removed for post mortem. Moribund or distressed birds were killed by carbon dioxide asphyxia. All birds remaining viable 2 weeks after infection were killed as above. Samples of liver, caeca and blood, where available, were taken using aseptic techniques and examined qualitatively for salmonellae using standard techniques as previously described [10, 11].

Laying hens

Two experiments were performed using laying hens. In the first, Rhode Island Red SPF birds (Compton) were obtained at 10 weeks of age and housed individually until *c.* 50% of the birds had started to lay eggs. The hens were then divided into two groups of 14 birds ensuring that each group contained an approximately equal number of birds in lay. Before infection, the two groups of birds were placed in separate rooms with each bird being caged individually. The hens were infected with *c.* 10^8 cells of Enteritidis PT4 isolate E or I by direct intubation into the crop. Birds were inspected at least daily. Faecal samples or cloacal swabs were collected at regular intervals and cultured for salmonellae using enrichment in selenite followed by selective plating. Eggs were removed as soon after lay as possible and stored in a separate room at room temperature until

the contents were cultured for salmonellae using previously published techniques [12]. Two weeks after infection, the hens were killed by carbon dioxide asphyxiation. Samples of blood, liver, spleen, ovary, oviduct and faeces were collected using aseptic techniques and examined qualitatively for salmonellae using previously published techniques [11].

In the second experiment, Hisex Brown commercial laying hens were used. The experimental protocols were as described above with the exception that there were 16 birds in each experimental group. With both experiments, the birds were provided with irradiated (5 Mrad) feed (SDS, Witham, Essex) and water *ad libitum*. During rearing and before infection faecal samples or cloacal swabs were taken at regular intervals and cultured for salmonellae using standard enrichment and plating techniques.

Survival in aerosols

Log and stationary phase broth cultures of the two Enteritidis PT4 isolates were standardized as described previously [2]. Aerosilization of bacterial suspensions and collection of samples were as described by Hambleton and colleagues [13]. Briefly, a rotating Goldberg drum was filled with an aerosol of a bacterial suspension generated by a Henderson apparatus at 65% relative humidity. Such a drum can maintain bacteria in aerosols for up to 24 h. Samples were drawn through 10 ml buffered peptone water at regular time intervals and a viable count (observed count) was performed on the samples by preparing serial, 10-fold dilutions 100 μl aliquots of which were plated onto nutrient agar. Viable counts were also performed on the bacterial suspensions before (SI) and after (S2) aerosolization. An initial sample was taken after 2 or 5 min to provide a reference viable count. Since a known volume of air was withdrawn from the drum and replaced with a water aerosol at each sample time, it was possible to calculate, from the reference count, the expected viable counts due only to dilution effects. The observed to expected ratio gave a measure of loss of viability due to being maintained as an aerosol. Two separate experiments were performed. One used log phase cultures and the other stationary phase cells.

Statistical analysis

The significance of differences between the two Enteritidis PT4 strains was examined using the χ^2 programme on Epinfo Version 6.

RESULTS

Observations on colony morphology of Enteritis PT4 isolates

Growth rates of isolates E and I on blood agar at 37 °C, as measured by increase in colony size, were very similar. If plates were then left at 20 °C, however, differences in colony morphology became apparent. Colonies of I, the chicken isolate, remained essentially round and shiny. In contrast, those of isolate E, from a human case, became 'wrinkled' in appearance and, after *c.* 48 h at 20 °C, resembled a colony of *Bacillus* spp. They also became progressively more difficult to emulsify in either saline or distilled water. If 'wrinkled' colonies were sub-cultured onto blood agar and incubated at 37 °C the subsequent growth resembled that of 'typical' salmonella colonies. If they were then left at 20 °C, reversion to a 'wrinkled' type occurred once more.

Salmonella-status of animals before artificial infection

All faecal samples or swabs from all animals tested before infection were salmonella-negative as assessed by direct culture in selenite broth followed by selective plating.

Virulence of the Enteritidis PT4 isolates in mice

The Enteritidis PT4 isolates showed marked differences in the virulence for the C57/BL6/J strain of mouse. In each of the three experiments significantly more mice died when infected with the human isolate E compared with those receiving chicken isolate I. The mean mortality ($\pm$S.E.) in the three experiments for isolate E was $82 \pm 15{\cdot}3$% and $10 \pm 4{\cdot}2$% for isolate I ($\chi^2 = 62{\cdot}06$; $P = < 0{\cdot}00001$). Data from the three experiments are shown in Figure 1. Culture age appeared to have no impact on virulence. Expts 1 and 2 were performed using 15 h cultures, whereas Expt 3 used a 3-h culture. There was quite good agreement between experiments although isolate E showed lowered virulence in Expt 2, where only 9/20 (45%) of the mice died (Fig. 1).

The mice receiving isolate E quickly became ill showing ruffled coats, eye rings and hunching. Their movements became increasingly slow and the majority of animals died or were killed within 6 days of infection. Where possible, moribund mice were killed to prevent further suffering. A few of the animals receiving isolate I died, although the majority (90% of

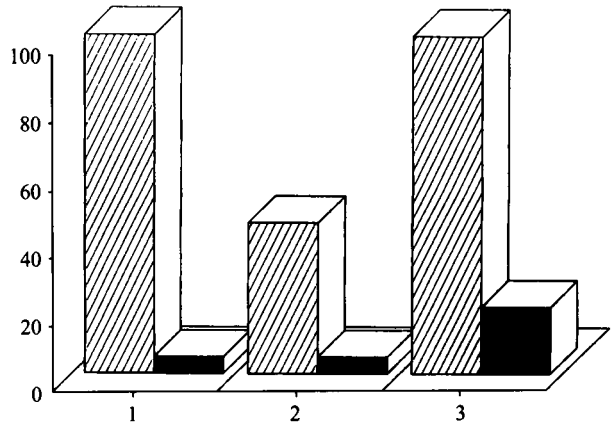

Fig. 1. Mortality in mice infected with different isolates of Enteritidis PT4. ▨, mice given human isolate E; ■, mice infected with chicken carcase isolate I. C57/BL6/J strain of mice infected by direct intubation into the oesophagus.

animals in the three experiments) remained well with only a few exhibiting obvious clinical symptoms.

Data from the cultures of tissues in the three experiments have been combined and are shown in Table 1. As to be expected, all the tissue samples from the mice which had been infected with isolate E, and which either died or were killed to prevent suffering, were salmonella-positive as were those from the few similar mice which had received isolate I (Table 1). There was, however, a marked contrast in the culture results from the mice which survived until the end of the experiments, 14 days post-infection. Almost all (87%) of the liver and spleen samples taken from surviving mice infected with PT4 isolate I were salmonella-positive, whereas none of the 11 samples from E mice yielded Enteritidis (Table 1). The latter data for mice infected with E were obtained from Expt 2 where only 45% of mice receiving this isolate died.

Infection of day-old chicks

The results from the two experiments were essentially the same and have been combined to give the data shown in Table 2. The two isolates behaved in a similar manner in the Hisex Brown chicks. During the course of the two experiments only 6/40 birds infected with E and 5/40 infected with I died. The rest of the birds remained essentially well. Invasion of the liver and colonization of the caecum were high with both isolates. There was some difference, however, in the isolation rates from blood taken from chicks killed at the end of the 2-week experimental period. With birds infected with E, 6/34 samples (18%) were salmonella-positive, whereas only 2/35 samples (6%) yielded salmonella from birds which had received I. However, differences were not significant.

Both PT4 isolates were able to infect control chicks held in a pen next to the infected birds. One bird (of 10) died in the group which were next to those which had been given isolate E. That apart, the other chicks in this control group remained well as did all those adjacent to the birds infected with isolate I. At post-mortem, 14 days after infection, all caeca from these birds were salmonella-positive. In addition, all chicks placed in the same room as those that had received isolate E had salmonella-positive livers as did 7/10 of those birds in with chicks infected with I. PT4 was also isolated from 2/9 (22%) and 1/10 (10%) of blood samples from E and I control birds respectively.

Isolation of Enteritidis from the tissue of laying hens

Two separate experiments were performed. SPF hens were used in the first and Hisex Brown commercial birds in the second. In both experiments, birds were infected with either PT4 isolate E or I by direct intubation of *c.* 10^8 cells into the crop. All birds remained clinically well during the 14-day period after infection and no differences were observed between the two groups. At post mortem, however, the prevalence of tissue invasion differed between the two PT4 isolates, particularly with regard to the positivity of reproductive tissues. The data from the two experiments are shown in Table 3.

Despite the fact that two different breeds of laying hen were used, the results from the two experiments show much similarity, although the Hisex Brown birds would appear to be better able to clear infection. Both PT4 isolates were invasive and although all blood samples were salmonella-negative, Enteritidis was recovered from a high percentage of liver and spleen samples (Table 3). The birds which had received isolate E yielded more salmonella-positive liver and spleen samples than those infected with I (Table 3) but the differences were of either low or no significance. In contrast, highly significant differences were noted in the ability of the isolates to invade reproductive tissue, particularly that of the oviduct. In Expt 1, using SPF hens, 13/14 (93%) of oviduct tissue samples were salmonella-positive in birds infected with E compared with 6/14 (43%) from birds infected with I ($\chi^2 = 9$; $P = 0{\cdot}0064$). In Expt 2, the corresponding figures were 8/14 (57%) with E and 1/15 (7%) with hens infected

Table 1. *Isolation of Enteritidis from the tissues of C57/BL6/J mice infected with PT4 isolates E or I*

PT4 isolate	Mice	No. samples from which salmonella were isolated*/no. examined (%) Liver	Spleen	Blood
E	Alive at 14 days†	0/11	0/11	0/11
	Died during experiment	42/42 (100)	42/42 (100)	20/20 (100)
I	Alive at 14 days	47/54 (87)	48/54 (87)	5/54 (9)
	Died during experiment	3/3	3/3	2/2

* It was not possible to get samples of blood, liver or spleen from all the mice which died during the experiment.
† These data are from Expt 2 only as in Expts 1 and 3 all the mice receiving isolate E died.

Table 2. *Isolation of Enteritidis from the tissues of Hisex Brown chicks infected with PT4 isolates E or I*

PT4 Isolate	Chicks	No. samples from which salmonella were isolated*/no. examined (%) Liver	Caeca	Blood
E	Alive at 14 days	34/34 (100)	33/34 (97)	6/34 (18)
	Died during experiment	6/6 (100)	6/6 (100)	2/2
I	Alive at 14 days	29/35 (83)	35/35 (100)	2/35 (6)
	Died during experiment	2/2	2/2	1/1

* See appropriate footnote at the bottom of Table 1.

Table 3. *The isolation of Enteritidis PT4 from the tissues of infected laying hens*

Tissue	No. samples from which salmonella were isolated*/ no. examined (%) SPF Hens PT4 isolate E	SPF Hens PT4 isolate I	Hisex Brown Hens PT4 isolate E	Hisex Brown Hens PT4 isolate I
Liver	14/14 (100)	8/14 (57)	4/15 (27)	3/16 (19)
Spleen	13/13 (100)	14/14 (100)	12/15 (80)	11/16 (69)
Blood	0/14	0/14	0/16	0/16
Ovary	11/14 (79)	6/13 (46)	3/14 (21)	1/15 (7)
Oviduct	13/14 (93)	6/14 (43)	8/14 (57)	1/15 (7)
Caecum	14/14 (100)	10/14 (71)	8/15 (53)	6/16 (38)

* See footnote for Table 1.

with I ($\chi^2 = 9{\cdot}4$; $P = 0{\cdot}0047$). Differences in the isolation rates from ovaries were not significant with P values of 0·09 and 0·27 in Expts 1 and 2, respectively.

Most birds remained faeces-positive for salmonellae throughout the course of the 14-day experimental period. It is of interest, however, that in Expt 2, using commercial Hisex Brown hens, 7/16 (44%) of birds infected with isolate I had salmonella-positive tissues although Enteritidis was not isolated from the caecum. The difference was even more marked in this group

Table 4. *Production of shell eggs by hens infected with Enteritidis PT4 isolates*

Expt no.	Bird type	PT4 isolate	Parameters measured: % birds laying at infection	% birds laying at slaughter	No. eggs laid while infected
1	SPF	E	43	86	70
		I	50	79	81
2	Hisex	E	63	93	149
		I	75	88	141

when tissue isolation rates were compared with the results from cloacal swabs. Isolate I was recovered from only one swab, whereas 13/16 (81%) birds were positive for salmonellae in liver, spleen or reproductive tissue. Very similar results were obtained from birds infected with isolate E.

Contamination of egg contents with Enteritidis and the impact of infection on egg-laying

Neither of the two Enteritidis PT4 isolates had any impact on the productivity of the infected hens. Data on egg-laying before and after infection are presented in Table 4.

Despite the observed differences in colonization of the reproductive tissues no significant differences were found in the contamination of egg contents with Enteritidis. In the first experiment, three birds which had received isolate E laid three eggs with salmonella-positive contents. One egg was clean while the shells of the other two were lightly soiled with faeces. Enteritidis was present in pure culture in each of these eggs. Two hens from the group infected with I laid seven salmonella-positive eggs between them. These eggs were more heavily contaminated with faeces and only two yielded Enteritidis in pure culture from the contents despite the fact that shells had been surface sterilized before contents were cultured. In Expt 2, which used commercial birds, one egg was contents-positive from each experimental group. The egg produced by the hens given isolate E was laid 6 days after infection. It had a clean, dry shell and Enteritidis was in the contents in pure culture. The egg from the I group was laid on the day of infection and was cracked and heavily contaminated with faeces. Enteritidis was present in the contents as part of a mixed population of coliforms.

Survival in aerosols

Enteritidis PT4 isolates E and I showed marked differences in their ability to survive in aerosols in both log and stationary phase. The human isolate E was capable of prolonged survival whereas isolate I died rapidly, particularly in log phase. Data from the two experiments are shown in Figure 2.

DISCUSSION

For Enteritidis to be transmitted from an infected laying hen to humans, for example, it may be necessary for the bacterium to possess certain characteristics. Thus, while salmonellae may be able to contaminate egg contents by invasion through the shell [14] infection of the reproductive tissue is a more important prerequisite for egg contents contamination [15–17]. The position of the bacterium within the egg, on or next to the yolk membrane, would suggest that infection of either the upper oviduct [17] or ovary is particularly important. When eggs are used as food it may be necessary for Enteritidis to be able to survive cooking and/or acidification whether in certain foods or the stomach. Previous work at this laboratory [2] demonstrated that isolates of PT4 differed in their tolerances of heat, acid, H_2O_2 and in their ability to survive on surfaces. The tolerant isolates were from human cases, whereas those that were more sensitive had been recovered from either chickens or eggs. This observation may suggest that human-derived PT4 strains, with a proven ability to cause infection, may possess attributes not found in other salmonellae. This prompted an investigation into behaviour of two isolates: E, from a human case and with high tolerance to the stresses mentioned above, and I, from a chicken carcase. The latter bacterium was particularly sensitive to heat, acid and H_2O_2 and survived very poorly on surfaces. Mouse and chicken models were chosen for these investigations.

A great many studies have used mouse models for the investigation of salmonella pathogenicity. Recent work [8–9] has confirmed earlier studies showing that there are differences in susceptibility between mouse strains. The C57/BL6/J mouse strain is clearly useful for differentiating between virulence of salmonellae and the results presented in this paper demonstrate that the human PT4 isolate E was substantially more virulent for this strain of mouse. Almost all the mice infected with this isolate died rapidly after infection or

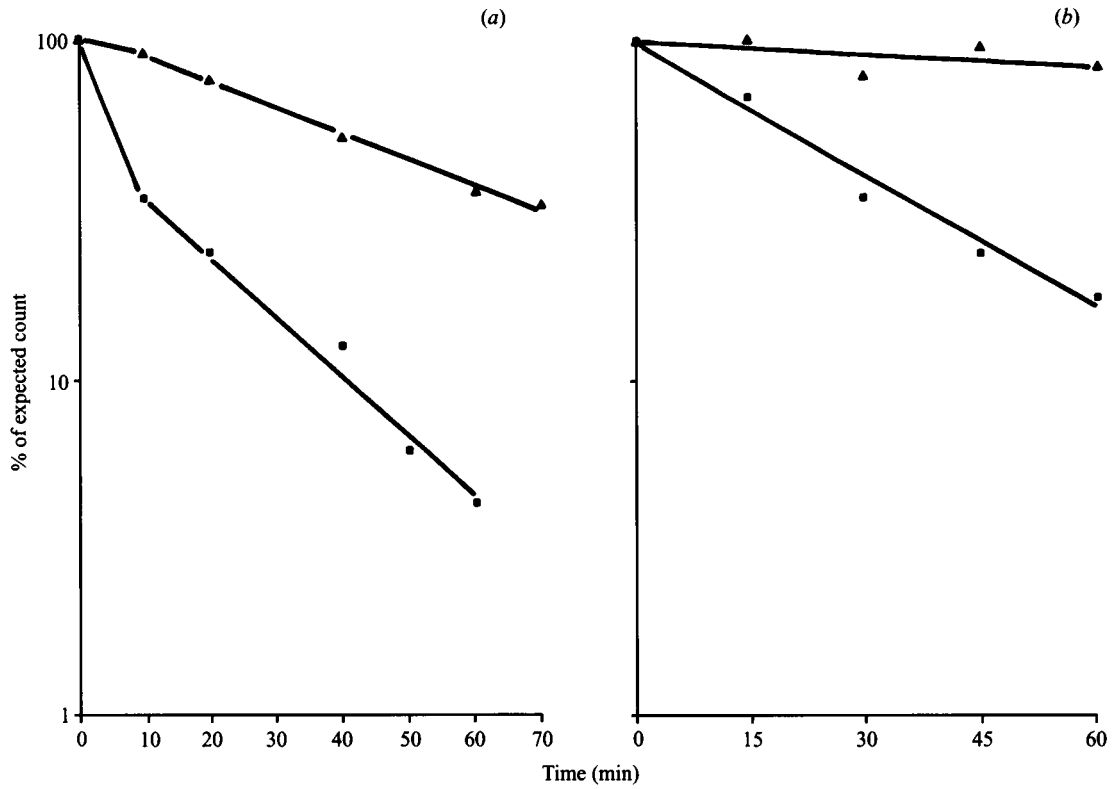

Fig. 2. Survival of Enteritidis PT4 isolates in aerosols. Isolate E, ▲; isolate I ■. (*a*) Log phase (3 h) cultures. (*b*) Stationary phase (15 h) cultures. For details of isolates see legend to Fig. 1.

were killed *in extremis* to prevent further suffering, whereas the great majority of mice receiving isolate I remained well.

In contrast to the data with mice, no significant differences were observed when the chicks of a commercial layer strain were infected by intubation into the crop. Both PT4 isolates were highly invasive, as measured by culture of liver tissues, but the birds remained well. This has been noted by other workers using oral infection in chicks with these two Enteritidis strains (Nagy, personal communication) and may reflect the relative PT4-tolerance of egg-producing chicken strains compared with those used for meat production [18]. It is possible that we were unrealistic to expect gross differences in either mortality or morbidity given the observations of other workers [5–7]. A better approach would be to use quantitative techniques and recent work [3, 4] has revealed Enteritidis PT13a variants that yield different numbers of bacteria from spleens in infected chicks. This technique will be used in all future studies.

Previous work at this laboratory has demonstrated that systemic PT4 infection can be achieved in chickens with a low infective dose by either the conjunctival route [11] or with aerosols [10]. This will be explored in future work with isolates E and I. Both were able to spread to chicks in adjacent pens but slightly higher infection rates, particularly with regard to bacteraemia, were seen with the E control birds. Given the much greater survival of this isolate in aerosols (Fig. 2) this result was perhaps to be expected.

Both PT4 isolates were highly invasive in SPF Rhode Island Red or Hisex Brown hens and, for example, a high proportion of liver and spleen samples were salmonella-positive (Table 3). Similar results were reported when a variant of Enteritidis PT13a which has the ability to contaminate egg contents at high frequency [5] was used to infect laying hens [19]. In this present study there were essentially no differences between either PT4 isolates or breed of bird in the infection of liver or spleen or colonization of the caecum and examination of these tissues may not be the best way to assess virulence in chickens. Infection of reproductive tissue is of far greater importance to the public health. Enteritidis PT4 isolate E would seem to differ from isolate I in its ability to infect reproductive tissue. Isolation rates from ovaries were higher in both experiments with birds infected with this isolate although the differences from I were not significant. The oviduct has been suggested as being of particular importance in the contamination of egg contents [15–17] and studies

with naturally infected laying hens revealed higher isolation rates from these tissues than from ovaries [17]. It is of interest, therefore, that isolation rates from oviduct samples were significantly higher, in both experiments, with the hens given isolate E. The infection rates in reproductive tissues of the hens infected with this isolate bore a close resemblance to those reported recently for a virulent Enteritidis PT13a isolate [19]. There were also other similarities between the two studies in that the rates of positivity of reproductive tissue greatly exceeded that of egg contents. The authors of the American study [19] postulated that many of the salmonellae present in the forming eggs are killed by the anti-bacterial properties of egg white. There would also seem to be variations in survival rates in these eggs both between and within salmonella serotypes [Keller, personal communication]. Egg contents contamination may also require the involvement of other factors such as stress which has been shown to affect oviduct tissues [20].

It was anticipated that differences would have been observed between the two isolates in egg contamination rates. This did not occur and, as discussed above, it may be that contamination of egg contents may require factors other than the presence of the bacterium in reproductive tissue or that isolate E is not maximally invasive. Egg contamination rates of *c.* 3% in this experiment are in line with studies on naturally infected hens [16] and those artificially infected into the crop [21]. They are substantially lower than those seen with hens infected intravenously [3] and thus how the bird acquires the bacterium may also influence egg contamination rates. It has also been suggested [19] that egg contents may be contaminated by Enteritidis cells moving up the oviduct from the cloaca and a recent study with Enteritidis PT13a reported a good correlation between faecal carriage and egg contents-contamination [19]. This was not seen in this present study nor in previous work with Enteritidis PT4 [10–11, 21].

All hens remained well during the course of the two experiments. This reflects the natural situation and once again indicates the possible difficulties in assessing the infection status of laying birds. This is exacerbated by the fact that Enteritidis PT4 is readily extra-intestinal. For example, in the experiment using Hisex Brown hens, 27/31 (87%) tissue samples were salmonella-positive for either isolate E or I, whereas only 4/31 (13%) of cloacal swabs yielded Enteritidis ($\chi^2 = 34{\cdot}1$; $P < 0{\cdot}001$). Such differences in isolation rates have been noted with naturally infected breeder flocks [22].

The principal purpose of the studies reported in this paper was to determine whether apparently closely related isolates of Enteritidis PT4, which have markedly different survival and tolerance profiles [2], also behave differently in animal models. The results demonstrate unequivocally that this is the case, particularly in the C57/BL6/J strain of mouse (Table 1; Fig. 1), although significant differences were noted in the ability to colonize parts of the reproductive tract of laying hens (Table 3).

It has been shown that the presence of a 54-kb plasmid encodes for virulence for mice in Enteritidis PT4 [23] although a different strain of mouse was used from the one in the investigations reported in this paper. It has also been proposed that in BALB/C mice virulence of PT4 is linked to the possession of a 38-kb plasmid which may encode for the ability to produce long chain LPS [24] although later work suggested that other factors may be more important [8–9]. The large amount of work involved in animal studies of the sort described in this present paper meant that it was only possible to examine two isolates in detail. However, previous work has shown that the phenotypic characteristics of these strains are typical of many within Enteritidis PT4 [2]. The differences in tolerance and survival are stable. Recent experiments with other PT4 isolates with phenotypic resemblance to E and I and which were isolated in either the UK, Australia or Germany also showed differences in virulence with a heat/acid-tolerant isolate being more virulent (Cox and colleagues, unpublished).

Both isolates E and I contain the virulence plasmid and LPS (unpublished data). Preliminary investigations indicate that the Enteritidis phenotype associated with strains like E is characterized by lipopolysaccharide enriched in O-antigen (Guard-Petter and colleagues, unpublished). The observation that growth phase appears to have no impact on virulence in C57/BL/J mice may mean that it is linked more closely to enhanced tolerance to H_2O_2 or better survival on surfaces as these attributes are largely unaffected by growth phase [2] unlike either heat or acid tolerance [2].

It is, of course, possible that the association between enhanced tolerance and greater virulence is coincidental and this is being investigated. It has yet to be determined whether there is a clear-cut distinction between human and avian PT4 strains with regard to tolerance to stressful conditions. It is interesting to speculate whether passage through human cases brings about altered tolerance or virulence. Current

work on isolates from cases and foods from the same outbreak will address this issue. Data to date (unpublished) indicate that there is no difference in either heat or acid tolerance in these isolates.

The results presented in this paper may have practical consequences and there is a need to be able to differentiate between virulent and avirulent strains of salmonella without necessarily resorting to the use of animal models. Previous work on Enteritidis PT13a isolates with different abilities to contaminate the contents of intact shell eggs in infected hens demonstrated that the isolate with a high contamination potential could be identified from avirulent isolates using colony immunoblots and antiserum specific for the factor 9 epitope of O-antigen as primary antibody [3]. In this study, differences in colony phenotype were also noted in that isolate E formed 'lacy-type' colonies, whereas isolate I did not. Thus, there is the possibility that colony morphology may be linked to virulence in Enteritidis [3–4, 25].

No attempt has been made in this study to elucidate the physiological or cellular factors behind the observed differences in behaviour of the two PT4 isolates. In addition to the possession of certain plasmids [23–24] or the production of long chain LPS [5–6, 8], possession of certain fimbriae may also be important [26–28]. Studies with a variety of potentially pathogenic bacteria [29] have demonstrated that virulence may be linked to an ability to respond to certain environmental stimuli within the host. These include change in temperature or pH. In addition to enhanced heat and acid tolerance [2] isolate E is also able to acid habituate in the presence of certain organic acids, whereas isolate I cannot [unpublished]. There is thus the possibility that the greater observed virulence of the human isolate E is linked to a capacity to respond more effectively to host-associated environmental signals.

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