Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella typhimurium* DT104

Steve A. Leach a*, Ann Williams a, Angela C. Davies a, Jenny Wilson b, Philip D. Marsh a, Tom J. Humphrey b

a Pathogen Microbiology, Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, UK
b PHLS Food Microbiology Research Unit, Church Lane, Heavitree, Exeter EX2 5AD, UK

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Abstract

Commercial laying hens were infected with *Salmonella typhimurium* DT104 strain 16 alternatively via the crop (10⁷ cfu per bird) or by an aerosol delivered directly to the beaks using a Collison nebuliser and Henderson apparatus (2 × 10² or 2 × 10⁴ cfu per bird). Infection by both routes caused systemic infection and prolonged contamination of faeces. Contamination rates of eggs and muscle were much higher following the aerosol challenges despite the much lower doses given by this route. The frequency of *Salmonella* isolation from eggs rose from 1.7% following oral challenge to 14% and 25%, for each of the aerosol challenges respectively, and the frequency of isolation from muscle rose from 0% following the oral challenge to 27% following each of the aerosol challenges. © 1999 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Reported cases of human salmonellosis caused by a multi-antibiotic-resistant strain of *Salmonella typhimurium* definitive type (DT) 104 have increased markedly in England and Wales over the last few years. The epidemic spread of antibiotic resistant strains is such that 95% of human isolates of *S. typhimurium* DT104 are now resistant to at least five antibiotics [1], with a similar increase in multi-resistant DT104 being reported in the USA [2].

Although numbers reported in England and Wales fell in 1997, they had reached 3996 cases in 1996 (P.G. Wall, personal communication).

Whilst DT104 is most commonly associated with cattle in the UK [3], a case-control study has identified a number of food vehicles implicated in DT104 infection including eggs and poultry meat [4]. Indeed, it has been demonstrated that commercial laying hens are highly susceptible to systemic infection by DT104 following experimental oral inoculation [5]. This resulted in a higher proportion of intact eggs being infected with this pathogen than had previously been observed with *Salmonella enteritidis* PT4 [5–10], which is of concern since intact shell eggs are...
the principal vehicle responsible for the current pandemic of PT4 infection [11].

*S. enteritidis* PT4 has been shown to infect poultry [6] and *S. typhimurium* to infect calves [12] when delivered experimentally by aerosol. Airborne contamination by *S. enteritidis* more generally has also been demonstrated to lead to the cross-infection of birds sharing the same rearing environment [13,14]. The aims of this study, therefore, were to determine specifically for *S. typhimurium* DT104 whether the aerosol route was effective in causing infection of commercial laying hens, and to compare the poultry meat and egg contamination rates following infection with measured doses of the pathogen by the oral and aerosol routes.

2. Materials and methods

2.1. Bacterial strain

*S. typhimurium* DT104 strain 16 (resistant to three antibiotics; tetracycline, sulfamethoxazole and streptomycin) was kindly supplied by the Veterinary Investigation Centre, Starcross, Devon. For infection of chickens, the strain was grown in and harvested from nutrient broth as previously described [10].

2.2. Oral infection of chickens

Point-of-lay Hisex Brown pullets (17 weeks old) were obtained from a commercial *Salmonella*-free source. Faecal samples were tested prior to infection to confirm that the birds were free of salmonellas. Fifteen birds were infected via the crop with 0.5 ml of culture diluted to contain approximately 10⁷ cfu of *Salmonella* (confirmed retrospectively) as described previously [10]. The birds were housed singly in cages following challenge.

2.3. Aerosol infection of chickens

Aerosols were generated using a Collison three jet nebuliser [15] in conjunction with a contained Henderson apparatus [16]. Two groups of 15 birds were screened as described above for *Salmonella* carriage and infected, as described previously, directly to the beak and into the lung [6], to give retained doses of approximately 10⁴ or 10⁵ cfu (confirmed retrospectively). Separate inocula which differed in bacterial cell density by a factor of 100 were aseptically prepared from the same broth. These were used to generate the two doses of aerosol via the nebuliser. Two birds from the higher dose group were killed immediately after infection, and the lungs removed for viable counts in order to determine the retained dose, which was 2.8×10⁴ cfu. The retained dose for the lower dose group was, therefore, calculated to be 2.8×10². Birds were housed singly in cages as before, with separate rooms for each dose following challenge.

2.4. Samples and microbiology

Birds were examined daily for signs of illness. Eggs were collected four times daily, and faeces collected every other day. After collection, eggs were aseptically transferred to sealed boxes pending bacteriological analysis. On day 14 post-oral infection, or day 13 post-aerosol infection, the chickens were killed and a blood and an entire pectoral muscle sample collected from each bird. Egg contents, faeces, blood and muscle tissue were examined for the presence of *Salmonella* using previously described methods [10].

2.5. Statistical methods

Statistical analysis of the results was performed, where appropriate, with either the chi-squared test or the Fisher’s two-tailed exact test (Statgraphics v 5.1, STSC, Maryland).

3. Results

Despite the much lower doses administered by the aerosol route, faecal excretion of DT104 was similar whether the challenge was given orally or by the aerosol route (Table 1). The proportion of faecal samples which were contaminated by DT104 were slightly lower immediately after inoculation by the aerosol route but soon reached similar or higher levels as those obtained with the oral inoculum, particularly at the higher aerosol challenge dose. The animals in the higher aerosol challenge dose also showed overt signs of illness, with some of the birds
being hunched, quiet and with ruffled feathers. One bird in this group was culled early for ethical reasons, 4 days after inoculation. The proportion of the birds which never had DT104 detected in their faeces was the same with the oral and lower aerosol challenge doses, being only 7% in both instances (Table 1). However, all of the birds had positive faeces at some point in the experiment following the higher aerosol dose. Blood samples taken at the end of the experiment which were *Salmonella*-positive were only obtained from a small proportion (7%) of the birds which had been given the higher aerosol challenge. Neither of the other two groups gave *Salmonella*-positive blood samples at the end of the experiment.

The most dramatic differences between the oral and aerosol challenges were in the rates of muscle and egg contamination (Table 1). The contamination rate for muscle samples rose significantly from 0% with the oral challenge to 27% following either of the aerosol challenges ($P = 0.038$, Fisher’s two-tailed exact test). The egg contamination rate also rose from 1.7% for the oral challenge to 13.8% and 25.4% for the lower and the higher aerosol doses, respectively. This difference between the aerosol and oral routes was highly significant ($P < 2 \times 10^{-7}$, chi-squared test). Although there was an increase in the egg contamination rate with the increased aerosol challenge dose this did not reach statistical significance ($P = 0.1$, chi-squared test). The bulk of the contaminated eggs following aerosol challenge were laid between day 4 and day 10 post challenge, with the rise in egg contamination following the higher challenge dose occurring about 1 day earlier (Fig. 1). By day 14, egg contamination rates had fallen, but were still higher than those seen in the birds infected orally, and remained higher in birds given the higher challenge dose. All but one of the birds given the higher aerosol dose laid DT104-positive eggs sporadically throughout the experiment, with one bird laying contaminated eggs daily.

### Table 1

<table>
<thead>
<tr>
<th>Route (dose, cfu)</th>
<th>Proportion of faecal samples contaminated (%)</th>
<th>Birds with <em>Salmonella</em>-negative faeces (%)</th>
<th>Number of <em>Salmonella</em>-positive eggs/total eggs</th>
<th><em>Salmonella</em>-positive eggs (%)</th>
<th><em>Salmonella</em>-positive muscle (%)</th>
<th><em>Salmonella</em>-positive blood (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral ($5 \times 10^6$)</td>
<td>93 87 87 87 80 73 67 7</td>
<td>3/178</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Aerosol ($2.8 \times 10^2$)</td>
<td>60 53 87 73 67 53 nd 7</td>
<td>20/145</td>
<td>13.8</td>
<td>27</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Aerosol ($2.8 \times 10^4$)</td>
<td>60 80 93 93 86 71 nd 0</td>
<td>32/126</td>
<td>25.4</td>
<td>27</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

*a* Negative throughout experiment.

*b* Data from [5]. nd, no sample since animal groups were culled on day 13.

*c* One bird was culled 4 days post infection.

![Fig. 1](image-url) Frequency of egg contamination from infected point-of-lay pullets over a 2-week period following aerosol route of challenge. ◇, $2.8 \times 10^2$ cfu retained aerosol dose; □, $2.8 \times 10^4$ cfu retained aerosol dose.
throughout the second week of infection. By contrast, in the lower dose group, four of the birds never laid Salmonella-positive eggs; contaminated eggs were laid sporadically throughout the experiment by the remaining 11 birds from day 4 onwards. Following oral infection, by contrast, DT104-positive eggs were laid only occasionally, one on day 4 after infection and two more 6 days later.

4. Discussion

*S. typhimurium* and *S. enteritidis* are both known to infect poultry and give rise to systemic infections in birds, leading to the contamination of poultry products and eggs [17]. Currently, *S. enteritidis* is the serotype most commonly linked to poultry-associated human disease, particularly phage type 4 in the UK, which steadily increased in prevalence from about 1982 to 1990. More recently, phage type 4 has also increased in prevalence in other parts of the world, including the USA, and PT4 is now considered to be a cause of a major poultry-borne pandemic [11].

The recent rise in reported cases of human salmonellosis caused by *S. typhimurium* DT104, and the demonstration, in some cases, of a link between human disease and eggs and poultry meat [4] suggest that DT104 has the potential to give rise to a new poultry-borne pandemic, although currently it is more commonly associated with cattle [3]. In earlier experiments, we demonstrated that DT104 could contaminate the contents of intact eggs laid by commercial hens following experimental oral infection [5]. The rate of contamination varied among the eight isolates tested but gave a mean egg contamination rate of 2.1% in the 14 days following infection. Muscle contamination, by contrast, was rare following oral infection, and was restricted to one strain which gave a low rate of contamination [5].

The airborne transmission of *S. enteritidis* and *S. typhimurium* within the rearing environment may have a significant impact on the cross infection of birds and the maintenance of these pathogens within poultry flocks. This has been demonstrated to be a potentially important factor in the cross infection of birds by *S. enteritidis* in experiments involving the passive movement of airborne contamination between spatially separated infected and uninfected birds [13,14]. This cross-contamination has also been demonstrated to be exacerbated by induced moulting [14]. Earlier experiments in which aerosols were generated experimentally had also shown airborne *S. enteritidis* to be very effective at infecting laying hens [6]. It is noteworthy that both *S. enteritidis* and *S. typhimurium* survive extremely well in these aerosols [18]; *S. typhimurium* survived better than *S. enteritidis* and showed no measurable decline in viability over two hours.

Given the capacity of *S. typhimurium* to survive aerosolisation and the potential importance of airborne cross-infection in poultry houses, we determined in this study the ability of *S. typhimurium* DT104 to infect laying hens by the aerosol route. Aerosols were generated experimentally using the Collison nebuliser and Henderson apparatus in order to deliver defined doses to the experimental animals and to compare the results with those derived from defined oral challenges of the same organism. Isolate 16 was used since this had previously given rise to a rate of egg contamination following oral challenge which was comparable to the mean of all eight of the DT104 isolates previously tested [5].

Birds were infected with equal efficiency by either route as determined by faecal excretion of the organism despite the 2 and 4 log lower doses administered to separate groups by aerosol. Although a small proportion of the birds never had DT104 detected in their faeces with the oral challenge, all of the birds had positive faeces at some point in the experiment following the higher of the two aerosol doses. This suggests a greater virulence of the pathogen when given by aerosol. Overt signs of disease were also only observed in the aerosol-infected birds, again suggesting greater virulence by this route. This was also suggested by the significantly higher rates of egg and muscle tissue contamination following the aerosol challenge compared to the oral challenge. The egg contamination rate rose from 1.7% to 14% and 25% depending on the aerosol dose, and the muscle contamination rate rose from 0% to 27% irrespective of the dose. The increase in muscle contamination which followed the aerosol challenges was paralleled by an increase in the number of blood samples that were *Salmonella*-positive. This strongly suggests that muscle positivity follows from septicaemia which in
many instances may be transient resulting in the muscle positivity
continuing well beyond the clearance of the pathogen from the blood. The bulk
of the contaminated eggs were laid between days 4 and 10 following aerosol challenge, and the contamination rate rose to about 70% of eggs laid during this period. A similar relatively short term rise in egg contamination rate has also been described following oral challenge by S. enteritidis [19]. However, following the higher aerosol challenge the contamination rate only declined to about 20% of eggs laid and remained at this level to the termination of the experiment.

The egg contamination rates following experimental oral infection with DT104 were already higher than had previously been found following equivalent experimental oral infection with S. enteritidis PT4 (0–0.8%) [5–10]. The egg and muscle contamination rates by DT104 following the aerosol route of infection were at least an order of magnitude higher again, which is of particular concern since this is considered to be a route of relevance to the poultry house environment. Collectively, these data provide support for the recent epidemiological finding that intact shell eggs may be a vehicle of human DT104 infection. The significantly higher levels of egg and muscle contamination with DT104 following infection by the aerosol route (at low infectious doses) may have profound implications for the egg and poultry meat industries if a similar epidemic to that caused by PT4 is to be avoided.

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References