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# Investigation of the parasitic nematode *Ascaridia galli* (Shrank 1788) as a potential vector for *Salmonella enterica* dissemination in poultry

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**Abstract** During recent years, the level of organically farmed poultry in Denmark has increased. Subsequent investigations have demonstrated an incidence of 64% of *Ascaridia galli* infections in layers established in organic farming systems. Studies to determine the interaction of *Salmonella enterica* with the parasitic nematode *A. galli* associated with poultry were undertaken to establish the significance of *A. galli* in the dissemination of *S. enterica*. *A. galli* was isolated from 40-week-old Lohmann Brown *Salmonella*-free layers. Worms were subsequently maintained in vitro and exposed to *S. e.* serovar Typhimurium at concentrations of $10^5$–$10^6$ colony forming units/ml for varying times (24–144 h). Eggs were harvested aseptically from the worms and the associations of *S. e.* Typhimurium in relation both to the eggs and to structures on the surface of the worm were studied, using immunofluorescence, viable counts and in situ hybridisation. Results show attachment of *S. e.* Typhimurium to the outer coating of the eggs and possible internalisation. Evidence of association of the bacteria with the nematode eggs was further substantiated by establishing *Salmonella* infection in day-old chicks after dosing them with eggs harvested from parasitic worms infected in vitro with *Salmonella*.

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## Introduction

The parasitic nematode *Ascaridia galli* (syn. *A. lineata*, *A. perspicillum*) occurs in the small intestine of the chicken, goose, guinea fowl, turkey and various wild birds in most parts of the world (Ackert 1931; Kates and Colglazier 1970; Soulsby 1982; Andersen 1992). The life cycle of *A. galli* is direct, involving two principle populations; the sexually mature parasite in the gastrointestinal tract and the infective stage ($L_3$) in the form of a resistant egg in the environment (Ackert 1931; Todd and Crowdus 1952; Araujo and Bressan 1977). The eggs are covered with three layers: the inner, permeable vitelline membrane, a thick resistant, shell and a thin, albuminous outer layer (Ackert 1931; Hansen et al. 1956; Cruthers et al. 1974).

Interactions between parasites and bacteria have been reported previously. However, studies involving the interaction of *A. galli* and the host bacterial flora are limited. A stimulating effect of the normal bacterial flora of the chicken on localisation and development of *A. galli* in the intestines of germ-free hosts has been demonstrated (Johnson and Reid 1973). This nematode develops favourably in the presence of monocultures of many Gram-negative bacteria; and *A. galli* and its host share identical intestinal flora but at a lower frequency in the former (Okulewicz and Zlotorzycka 1985). Only *Arizona* sp. appeared more frequently in the parasite intestine than in the host. The pH of both intestinal systems were also comparable, pH 6.1–7.0 for the chicken intestine and pH 6.1–6.7 for the parasite. Identical flora to that found in the intestine of *Ascaridia galli* was also seen on the surface of the cuticle. However, *A. galli* also demonstrated an inhibitory effect on the gut flora of its host, the extent of which was dependent on the prevalence of the parasites.

There have been previous reports suggesting the possibility of parasitic helminths acting as vectors and harbouring bacterial species. Neves et al. (1969) and Ottens and Dickerson (1972) demonstrated that *Schis-*

*tosoma* spp could harbour *Salmonella enterica* serovar Typhi and *S. e.* Paratyphi. Evidence of transmission of *Escherichia coli* via migrating *Strongyloids* larvae to tissue from the gastrointestinal tract was shown by Brown and Perna (1958), Wilson and Thompson (1964) and Walker-Smith et al. (1969). Bottjer et al. (1978) reported that the nematode parasite *Nematospiroides dubius* third stage larvae act as vector for *Salmonella enterica* serovar Typhimurium in the infection of mice. Nalin and McLaughlin (1976) demonstrated colonisation of *Ascaris lumbricoides* by *Vibrio cholerae*, and Adedeji et al. (1989) reported a synergistic effect of *Ascaris* larvae and *E. coli* in piglets.

Transmission of *Histomonas meleagridis* is intimately associated with the caecal nematode *Heterakis gallinarum* and several species of earthworms common to poultry yard soil (Tyzzer 1926; Lund 1958; Lund et al. 1966; Lund and Chute 1973; Calnek et al. 1997). The mechanism of egg infection in *H. gallinarum* by histomonads has, however, not been experimentally determined.

A cross-sectional prevalence study of gastrointestinal helminths in Denmark which included 268 adult hens, has been carried out (Permin et al. 1999). There was a variation in the number of species found and the prevalence between different production systems was apparent. *Ascaridia galli* demonstrated a prevalence of 64% in free-range/organic systems and 42% in deep-litter systems. Previously, table egg production has been based on battery cage and deep-litter systems. However, developments in consumer demands have resulted in increased production in free-range/organic systems. It was apparent from the study that such outdoor systems demonstrated a high prevalence of *A. galli* infections.

With the exception of minor incidences reported periodically, *S. enterica* has now been eradicated at the parent stock level from the confined broiler industry in Denmark, in which *A. galli* infections are also rarely reported (Permin et al. 1999). However, major problems have been defined in association with table egg production, in which the prevalence of *A. galli* infections are high (Permin et al. 1999).

This study looked at the potential of *A. galli* to act as an additional risk factor in the dissemination of *Salmonella* in chicken. It investigated the association of *Salmonella* with the parasite itself, but in particular with the nematode eggs which are readily excreted into the environment.

## Materials and methods

### Bacterial strains

A clinical isolate of *Salmonella enterica* serovar Typhimurium was used in the study. The strain was a rifampicin-resistant mutant ($rif^R$), which facilitated the enumeration of the organism in worms, worm eggs and, thereafter, in chicken faeces. Broth cultures were made in 10 ml LB broth, containing (per litre): 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco), 5 g NaCl, pH 7.7, 50 μg rifampicin/ml and were incubated aerobically at 37 °C, with shaking. Bacterial colony counts were adjusted to approx. $10^8$ colony-forming units (cfu)/ml using a spectrophotometer ($OD_{620} = 0.1$) and confirmed with viable counts on LA [Bacto tryptone (Difco) and 50 μg rifampicin/ml], prior to subsequent inoculation.

### Isolation of *Ascaridia galli*

*Ascaridia galli* worms were isolated from 40-week-old *Salmonella*-free Lohman Brown layers. The entire gastrointestinal tract, from the gizzard to the cloaca, was removed from the birds and carefully dissected to remove the worms. All birds were naturally infected. Infection with the parasite was determined prior to post mortem by counts of eggs per gram of faeces (Permin 1997).

### In vitro cultivation and infection studies with *A. galli*

Several cultivation media were initially tested for their efficacy to sustain survival of the parasitic worm in vitro. The media used were: (1) Hedon-Fleig solution [calcium chlorodihydrate (335 mg), calcium chloride (1.5 g), magnesium sulphate (1.5 g), disodium sulphate-dihydrate (2.5 g), glucose-monohydrate (5.0 g), sodium chloride (35.0 g) and distilled water (up to) 5 l], (2) RPMI 1640 with phenol red (Gibco, BRL), (3) Eagle's minimal essential medium without phenol red (Gibco, BRL), (4) Dulbecco's medium without phenol red (Gibco, BRL) and (5) dextrose-cornmeal agar (Pharmacia, Sweden) with saline. A total of 50 worms (10 male, 40 female) were incubated in 1 l medium. Each medium was examined either with or without addition of *S. e.* Typhimurium (approximately $10^6$ cfu/ml), corresponding to what has been seen in natural infections (Barrow et al. 1988). Survival and activity of the worms were then determined over a 6-day period using an arbitrary scale: + + vigorous activity, + sedate movement, ± minimal movement and – stationary/dead. The medium was changed every 24 h and replaced with pre-warmed fresh media, together with an appropriate inoculum of *S. e.* Typhimurium.

Worms were maintained at either 37 °C (aerobically and in an atmosphere of 5% $CO_2$) or 41 °C. Eggs from the worms were initially obtained by aspirating the bottom of the flasks containing the worms. However, this often resulted in the collection of unwanted debris. Subsequently, eggs for this study were obtained from female worms identified and rinsed briefly in 70% ethanol and distilled water prior to examination and dissection to avoid contamination. To harvest the *A. galli* eggs, the anterior and posterior uteri were dissected from each worm in saline solution, using sterile instruments and were subsequently transferred to distilled water. Uteri from four separate worms were pooled in 1.6 ml distilled water. The solution was then vigorously mixed to release the eggs from the uteri. Alternatively, eggs were harvested from the uteri and washed nine times, either by decanting (eggs were washed in 10 ml saline and allowed to settle for 30 min, after which the wash was decanted and replaced with 10 ml of fresh saline) or by centrifuging (a mixture of 10 ml of saline and egg suspension was centrifuged at 1,200 rpm for 7 min; and the supernatant was discarded and replaced by fresh saline). This procedure was implemented to further ensure that any *Salmonella* potentially contaminating eggs during preparation would be washed away. The number of washes was calculated to remove all possible contamination in the wash solution. An infective dose of eggs was then prepared. Microscopy and viable counts on the egg suspensions were made, together with an egg count. Day-old, *Salmonella*-free chicks received an oral dose of 100 ml of the infective dose.

### *S. e.* Typhimurium-specific screening protocols

#### *Light microscopy*

An aliquot of saline suspension with *A. galli* eggs was dropped onto a glass slide, allowed to dry and then fixed with absolute methanol for 5 min and air-dried again. The preparation was then stained

with Giemsa stain (E. Merck, Darmstadt, Germany) diluted 1:40 from the stock solution (azure-eosine-methylene blue solution) in distilled water for approximately 1 h (Murray et al. 1994). The slides were then rinsed in 95% ethanol to remove excess dye and air-dried before examination under oil immersion to identify bacterial cells associated with the surface structure of the eggs.

*Immunofluorescence staining*

Samples of whole worms and eggs were fixed in formalin. Thereafter, dehydration with a series of ethanol solutions (96–99%) followed by petroleum. Samples were then set in paraffin blocks for subsequent sectioning. An indirect immunofluorescence technique was applied (Jensen and Schønheyder 1989). Sections were dewaxed with xylol, rehydrated and rinsed with saline buffered with 20 m*M* Tris, pH 7.6 (TBS). Sections were treated with 0.1% trypsin (Sigma) in TBS for 30 min at 37 °C and then incubated with 5% normal pig serum (NPS) in TBS for 10 min at 20 °C. Polyclonal rabbit antiserum against *S. e.* Typhimurium (Pospischil et al. 1990) was diluted in NPS + TBS and incubated at 20 °C for 30 min with fluorescein-isothiocyanate-conjugated pig anti-rabbit immunoglobulin (Dakopatts F205, Denmark), diluted 1:20 in NPS + TBS. Sections were rinsed and mounted in 2.5% 1,4-diazobico-(2,2,2)-octane (Sigma) in glycerol, pH 8.6. Slides were washed in TBS and viewed under an epifluorescence microscope. Controls included pig lung tissue sections previously proved positive and sections without exposure to *S. e.* Typhimurium (Nordentoft et al. 1997).

*In situ hybridisation*

Samples were prepared as above; and hybridisation solution (0.7 *M* NaCl, 0.1 *M* Tris-HCl, pH 8.0, 0.1% sodium dodecyl sulfate, 10 m*M* ethylenediaminetetraacetic acid) containing 5 ng of fluorescent-labelled oligonucleotide probe targeting ribosomal RNA/μl (Nordentoft et al. 1997) was added. Slides of the sections were placed in a humidified chamber and incubated for 3 h at 45 °C. Sections were subsequently washed in cold (deionised) water ($ddH_2O$) and re-incubated in pre-heated hybridisation buffer at 45 °C for 20 min. Finally, the slides were rinsed in $ddH_2O$ and air-dried. The slides were mounted in a phosphate-buffered (pH 8.5) medium, containing 20 mg of *n*-propyl gallate/ml (Sigma Chemicals) to prevent fluorescence fading. The hybridised sections were visualised by epifluorescence microscopy at ×100 on a Leica DMRB microscope (Leica, Wetzlar, Germany) for excitation at 450–490 nm.

*In vivo studies*

Groups of ten 1-day-old *Salmonella*-free chicks were given infective doses (100 μl, orally) of uteri-harvested *A. galli* eggs, either directly or subsequently washed as stated previously. Association of *S. e.* Typhimurium with eggs was demonstrated prior to dosing by viable counts (serial dilutions in saline and plating on LA plates with 50 μg of rifampicin/ml), by light and fluorescent microscopy and by in situ hybridisation. There were four groups of five birds: group A received an inoculum of approximately 3,300 eggs with an associated challenge of $1.5 \times 10^5$ *Salmonella*, taken directly from the uteri of worms without washing, group B received approximately 3,000 eggs with $9.1 \times 10^3$ *Salmonella* (eggs were washed nine times by decanting prior to inoculation), group C received approximately 3,400 eggs with $3.4 \times 10^3$ *Salmonella* (washed nine times with centrifugation prior to inoculation) and group D was the control group, receiving approximately 3,000 eggs from *A. galli* not exposed to *S. e.* Typhimurium. The *Salmonella* excretion rate was monitored daily over 7 days, using cloacal swabs. Colonisation of caeca and liver at the end of the experimental period were determined by stomaching 1 g of faeces or liver tissue in saline solution (0.8%), followed by serial dilutions. All counts were carried out on LA plates with 50 μg of rifampicin/ml incorporated.

## Results

### In vitro cultivation of *Ascaridia galli*

Investigation of survival of the adult *Ascaridia galli* nematode in vitro was carried out with five different media. The two media providing most efficient cultivation were Hedon-Fleig and dextrose-cornmeal agar. Both of these media allowed survival of the adult worms for up to 6 days at 37 °C (Table 1). However, the final day demonstrated diminished activity of the worms, indicating imminent death. The measurement of survival was based purely on movement of the worms, which was assigned an arbitrary rating (Table 1, footnote; see also Materials and methods: In vitro cultivation and infection studies with *A. galli*). Of the three incubation conditions used, aerobic incubation at 37 °C was most suitable. This was more beneficial than aerobic incubation at 41 °C, where only 4 days survival was demonstrated.

Additional media tested (Eagles, Dulbecco's and RPMI 1640) were originally designed as tissue culture media for primary cells and cell-lines in vitro. These provided some degree of longevity of the worms; and aerobic incubation at 37 °C again provided the most suitable conditions. *Salmonella enterica* serovar Typhimurium was not inoculated into these media, due to the high nutrient level available and the potential for a profuse increase of bacterial growth from the original inoculum. (Bacteria have an approximate doubling time of 30 min in this medium.)

### Association of *S. e.* Typhimurium with *A. galli* eggs

Light microscopy of the *A. galli* eggs stained with Giemsa (Fig. 1) clearly demonstrated abundant bacterial attachment to the outer structures of the *A. galli* eggs. Although Giemsa staining is non-specific to *Salmonella*, the eggs were isolated from worms cultivated in vitro in a medium inoculated with *S. e.* Typhimurium. At this stage, it was evident that bacterial attachment alone could provide a means of disseminating *Salmonella* outside the bird, given the large number of bacteria present and their strong adherence. Immunofluorescent staining specific for *S. e.* Typhimurium demonstrated attachment to the integument of the eggs but did not emphatically prove internalisation of the bacteria (Fig. 2). Finally, *S. e.* Typhimurium was also demonstrated to be associated with *A. galli* eggs by in situ hybridisation. This was demonstrated a number of times with different preparations originating from separate worms.

### In vivo challenge of chicks with eggs aseptically isolated from *A. galli* and subsequently infected with *S. e.* Typhimurium

All three inoculations of eggs (A, B and C) demonstrated associated *Salmonella* counts, albeit at different

**Table 1** Determination of optimal in vitro cultivation and simultaneous infection of *Ascaridia galli* worms with *Salmonella enterica* serovar Typhimurium, indicated by activity/survival of *A. galli* worms. + + Vigorous activity, + sedate movement, ± minimal movement, – stationary/dead. *rif* Rifampicin (50 µg/ml)

| Medium | Incubation (temperature, hours) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 37 °C | | | | | | | 37 °C + $CO_2$ | | | | | | 41 °C | | | | | | |
| | 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | | 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | |
| Hédon-Fleig | | | | | | | | | | | | | | | | | | | | |
| + *S. e.* Typhimurium (+rif) | + | + | + | + | + | ± | | | | | | | | | | | | | | |
| (Without rif) | + | + + | + + | + | + | ± | | + | + | + + | ± | – | – | + | + | + | ± | – | – | |
| Without *S. e.* Typhimurium (+rif) | ± | + | + | + | + | ± | | | | | | | | | | | | | | |
| (Without rif) | ± | + + | + + | + + | + | ± | | + | + | + + | ± | – | – | + | ± | + | ± | – | – | |
| RPMI 1640 (+phenol red) | | | | | | | | | | | | | | | | | | | | |
| Without *S. e.* Typhimurium | + + | + + | + + | + + | ± | – | | + | + | + + | – | – | – | + | + | ± | – | – | – | |
| Eagle's (without phenol red) | | | | | | | | | | | | | | | | | | | | |
| Without *S. e.* Typhimurium | + + | + + | ± | – | – | – | | + | + | ± | – | – | – | + | + | ± | – | – | – | |
| Dulbecco's (without phenol red) | | | | | | | | | | | | | | | | | | | | |
| Without *S. e.* Typhimurium | + | + | + | ± | + | – | | + | + | + | – | – | – | + | ± | ± | – | – | – | |
| Dextrose-cornmeal agar + NaCl | | | | | | | | | | | | | | | | | | | | |
| + *S. e.* Typhimurium (+rif) | + + | + | + | + | + | ± | | | | | | | | + | + | ± | ± | – | – | |
| Without *S. e.* Typhimurium (+rif) | + + | + | + + | + | + | ± | | + + | + + | + + | ± | – | – | + | + | ± | ± | – | – | |

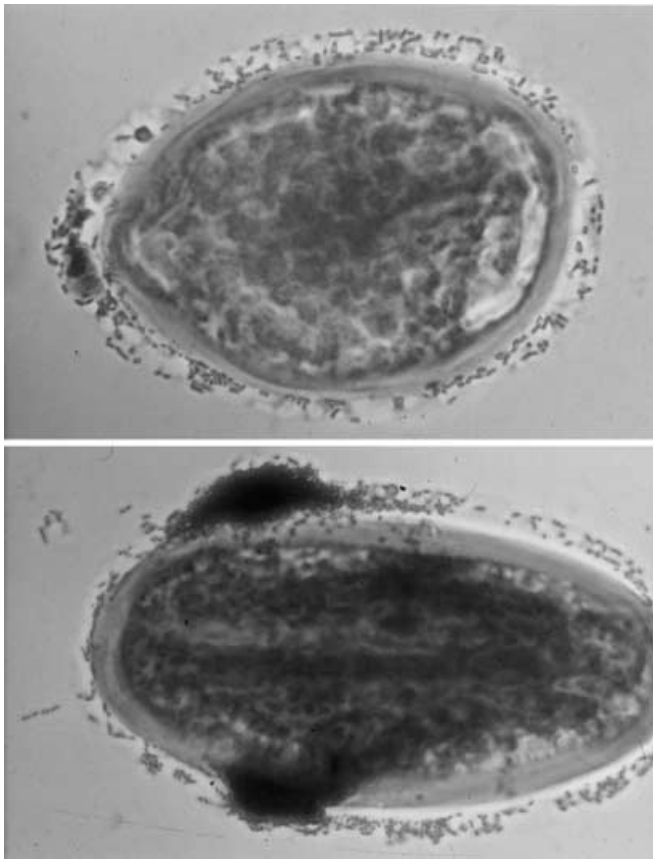
levels. Thorough washing of the eggs still demonstrated associated *Salmonella*, indicating either strong adherence of bacteria to the exterior coat or possibly internalisation. Bacterial counts from the individual washes were made on LA with rifampicin (50 µg/ml) and final washes gave no bacterial counts (data not shown).

Excretion of *S. e.* Typhimurium was demonstrated in all three groups throughout the 7 days, while the control group (D) remained negative (Fig. 3A). Groups A and B still showed heavy excretion on day 7; and colonisation of the caeca and livers of the respective groups is shown in Fig. 3B.



**a**

**Fig. 1** (Contd.)



b

**Fig. 1A, B** Demonstration of bacterial attachment to the outer coating of the *Ascaridia galli* egg around the oval structure. **A** Eggs were harvested from the uteri of adult female worms (Group A), incubated with *Salmonella* enterica serovar Typhimurium (in vitro, 37 °C, 3 days), subsequently stained with Giemsa and viewed under light microscopy (×100, oil immersion) in different planes. **B** Further evidence of attachment of bacteria to the albuminous "buffer coat" layer of the *A. galli* egg. Eggs were harvested by aspiration from media containing worms and *S. e.* Typhimurium, after release from the females. Giemsa staining and light microscopy (×100, oil-immersion)

## Discussion

A dextrose-cornmeal agar was originally reported for in vitro sustainability of *Ascaridia galli* (Ackert 1931). The Hedon-Fleig solution had already proved to be applicable in maintaining *Ascaris suum* worms outside the pig host (Pantelouris 1965; Eriksen 1981). As soon as in vitro sustainability of the parasite was achieved, attempts to infect the worms with *Salmonella enterica* serovar Typhimurium were pursued. Survival of the worms outside the host was possible for up to 6 days in the two minimal media of Hedon-Fleig and dextrose-cornmeal agar with saline. The incubation temperature providing more efficient survival was 37 °C and not

►

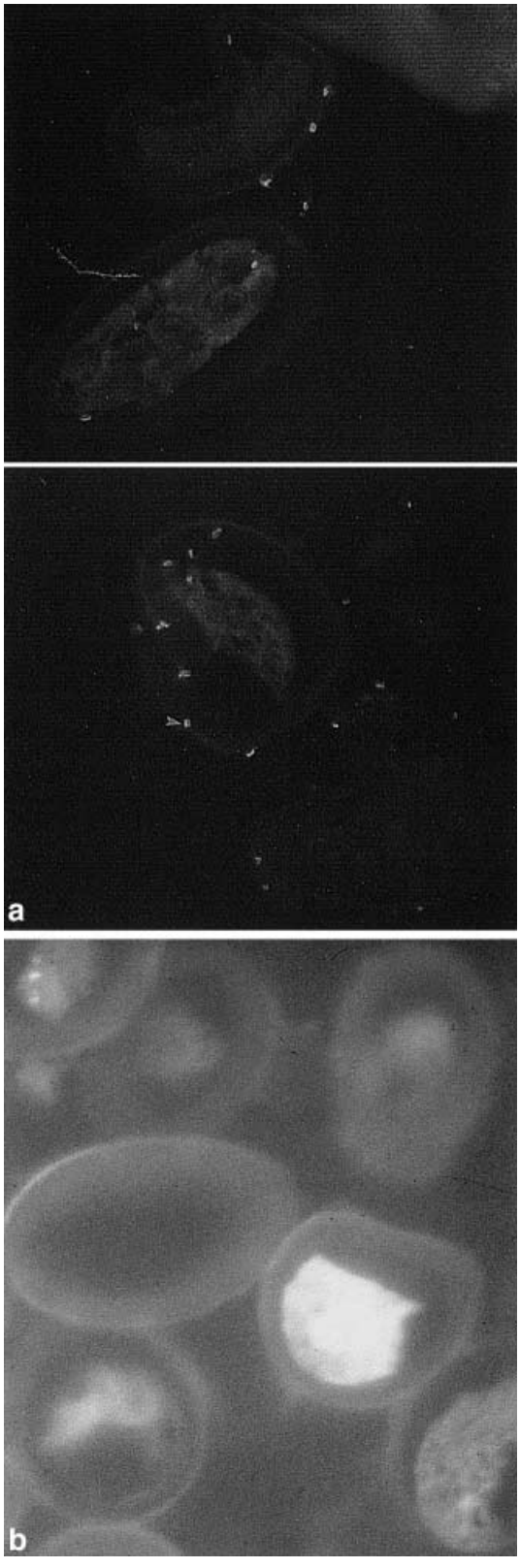



**Fig. 2A, B** *Salmonella*-specific staining of cross-sections of *A. galli* eggs obtained from the uteri of adult female worms (Groups A, B, C) previously exposed to *S. e.* Typhimurium. **A** Immunofluorescent staining specific for *S. e.* Typhimurium. *Salmonella* is attached to the integument of the eggs. Bacteria may be located internally but confirmation of this is not possible from these sections (UV microscopy, ×50). **B** Immunofluorescent staining specific for *S. e.* Typhimurium of eggs obtained from worms which were not cultivated with *Salmonella* (Group D). No *Salmonella* was detected

**(A)** Salmonella excretion.

| Group | Age of chicks (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | **4** | **6** | **7** |
| **A** | - | + | + | + | - | ± | ± |
| | - | - | + | + | ± | ± | + |
| | - | + | + | + | + | + | + |
| | - | + | + | + | ± | + | + |
| | - | + | + | + | ± | + | + |
| **B** | - | ± | ± | + | + | + | ± |
| | - | + | + | + | + | + | + |
| | - | - | - | + | + | ± | + |
| | - | - | - | - | - | - | ± |
| | - | - | + | + | - | - | + |
| **C** | - | - | + | + | ± | ± | ± |
| | - | + | + | + | ± | + | ± |
| | - | + | + | + | + | + | ± |
| | - | + | + | ± | + | ± | ± |
| | - | + | + | + | ± | + | ± |

+ growth >50 colonies ± <50 colonies of growth - no growth

**Group A** – Approx. 3300 eggs / bird with associated calculated challenge of $1.5 \times 10^5$ salmonella (cfu) [Eggs removed directly from uteri, not washed].

**Group B** – Approx. 3000 eggs / bird with associated calculated challenge of $9.1 \times 10^3$ salmonella (cfu) [Eggs washed by decanting, 9x].

**Group C** – Approx. 3400 eggs / bird with associated calculated challenge of $3.4 \times 10^3$ salmonella (cfu) [Eggs washed by centrifugation, 9x].

**Group D** – Control group, eggs from worms not infected with S. Typhimurium (approx. 3000 eggs) – all eggs were negative for S. Typhimurium prior to infection of the birds and consequently there was no salmonella excretion.

**Fig. 3** (Contd.)

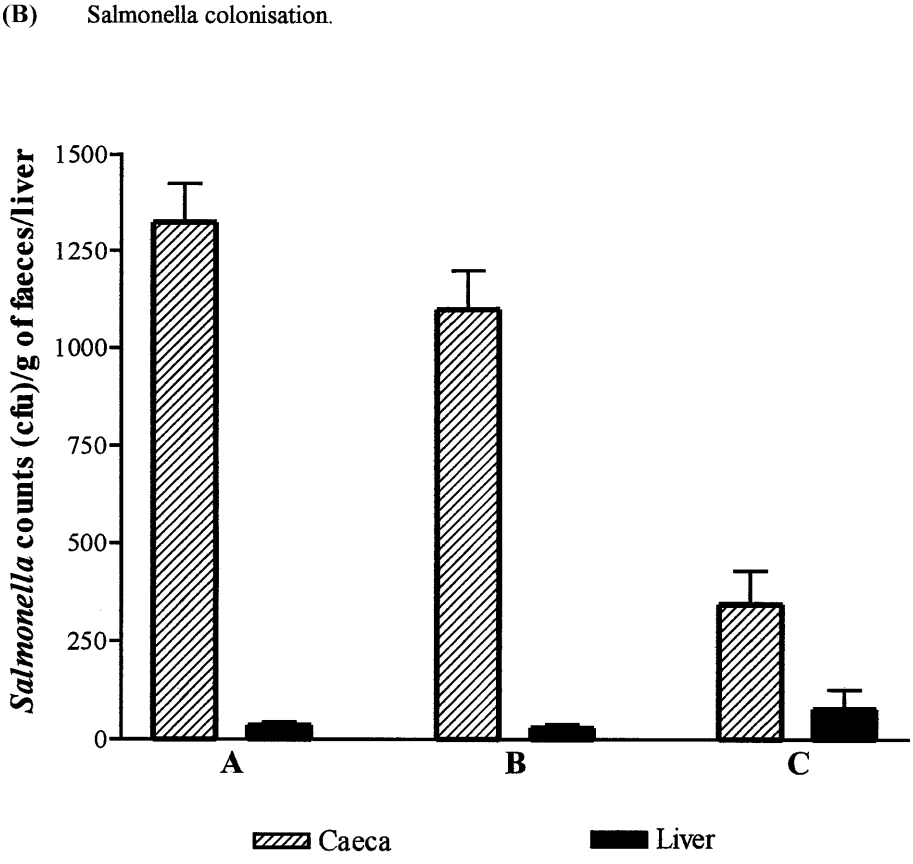



**Fig. 3A, B** Determination of *Salmonella* excretion rate and colonisation in chicks 1 week after challenge of *S. e.* Typhimurium-infected *A. galli* eggs. *cfu* Colony-forming units

41 °C which was suprising, given that the latter corresponded to the body temperature of the host chicken.

In addition to survival of the worms, the *Salmonella* inoculum introduced into the media was allowed to remain constant and viable (colony counts on LA plates with rifampicin (50 μg/ml) showed no significant differences from the original inoculum after 24 h incubation, results not shown), therefore, an accurate measure of the initial inoculum was maintained. It was observed that, in some instances, addition of the *Salmonella* actually increased worm activity. This may indicate some comparison to the natural environment of the parasite, where they are surrounded by the intestinal bacterial flora.

Initial work demonstrated abundant attachment of bacteria to the worms external cuticle (not shown). Subsequent work was concentrated on demonstrating the association of *S. e.* Typhimurium with the parasite eggs, which are readily excreted from the host into the environment. Eggs were originally harvested from the bottom of the flasks harbouring the worms and *Salmonella* and were examined on a daily basis. Figure 1B demonstrates extensive bacterial attachment to the eggs. This procedure, however, provided a limited number of eggs that were heavily contaminated with waste-products from the adult worms. In addition, using eggs harvested under these conditions did not exclude the possibility that the *Salmonella* originated from the growth medium. A refined technique of extracting the eggs directly under aseptic conditions from the worms was used instead. The reasons behind this were not only to obtain eggs free from debris and at a higher concentration but also to demonstrate that the infection of the eggs with *S. e.* Typhimurium took place within the worm. It was suggested that if the worm itself was infected with *Salmonella*, the eggs developing in the reproductive tract could be colonised via the vaginal opening allowing the entry of *Salmonella* onto/into the structure of the egg prior to the outer coating being fully formed. This was a reasonable assumption, given that (1) previous in situ hybridisation studies had indicated a positive association of bacteria with the structures of the *A. galli* eggs and (2) such a phenomenon already existed in nature, i.e. *S. e.* Enteritidis can infect the chicken by vertical transmission via the egg, infecting the reproductive tract of the chicken and establishing in the egg itself prior to shell formation (O'Brien 1988). However, other scenarios could be considered. Tyzzer (1932) suggested that *Histomonas meleagridis* reached the reproductive tract and eggs of the caecal worm *Heterakis gallinarum* by injection into the uterus of the female during copulation (originating from the intestinal tract of the male). In the male worm, both the alimentary tract and reproductive systems discharge through a common cloaca. This is also the case with the *A. galli* male worm. Niimi (1937) hypothesised that histomonads were taken up orally into *H. gallinarum* via the mouth, travel to the lumen and subsequently pass through the

gut wall, to appear in the body cavity. In the male they disappear readily, but in the female they go on to invade the ovaries and the eggs.

Eggs harvested aseptically from the uteri in the manner described above were treated in three separate ways: (1) released directly from the uteri into saline for subsequent inoculation of chicks, (2) harvested from the uteri and (3) washed several times by decanting or centrifugation. The first approach was aimed at limiting the removal of adherent salmonella and the other two methods allowed some degree of mechanical removal of attached bacteria from the outside of the eggs. Each preparation demonstrated the association of *S. e.* Typhimurium with the eggs and resulted in subsequent *Salmonella* infection of the chicks, confirming that eggs dissected from adult worms incubated in vitro with *S. e.* Typhimurium have *Salmonella* attached, with the possibility of internalisation. It also confirmed that consequent infection of day-old, *Salmonella*-free chicks with these eggs resulted in *Salmonella* colonising the birds. Repeated centrifugation and washing of the eggs prior to infection of the chicks failed to remove association with *S. e.* Typhimurium, although this procedure should enable the removal of up to $10^9$ organisms; and final washes were negative for *Salmonella*. This in itself demonstrates either a strong attachment of *Salmonella* to the exterior structures of the egg, or definite internalisation. The strong attachment is further indicated by the fact that repeated decanting/centrifugation and washing, designed to remove up to $10^9$ organisms, only reduced the *Salmonella* counts from $1.5 \times 10^5$ (group A) to $3.4–9.1 \times 10^3$ (groups B and C).

Previous studies of bacterial carriage via parasites have focused on determining internalisation of the bacteria and have exposed the worms to treatment either with hydrochloric acid (Nalin and McLaughlin 1976) or with sodium hypochlorite (Bottjer et al. 1978) to prevent bacteria from contaminating the external structures of the worms. Further studies could be developed to substantiate internalisation of *Salmonella* within the eggs of *A. galli* by pretreatment of the eggs with acid prior to dosing birds or physical decoating of the eggs, thus eliminating the possibility of infection via externally attached bacteria. A longer-term study could involve embryonation of the eggs to the infective $L_3$ stage in vitro using either 0.1 N sulfuric acid, 2% formalin or vermiculite (Permin et al. 1997) and infecting birds to allow development of the parasitic infection, followed by monitoring the *Salmonella* excretion rate and parasitic eggs in the faeces and finally post mortem examination for *Salmonella* colonisation and the presence of worms in the intestinal tract. This would verify whether a full cycle of *Salmonella* infection could be coupled with parasitic infection. However, results from this study have already shown that not only the nematode itself, but also the eggs were capable of harbouring salmonellae and, therefore, potentiated a risk of contamination with and spread of *S. enterica*. With a low inoculum of *Salmonella* associated with the nematode eggs, subsequent infection of day-old chicks was still possible. The parasite and bacterium may function together in a synergistic dual infection, in that infection of *Salmonella* from a low initial inoculum is possible, as seen in the study of Bottjer et al. (1978) with the nematode *Nematospiroides dubius* and *S. e.* Typhimurium in mice. *Salmonella* infection may also be potentiated in the presence of *A. galli* infections. It is also apparent that *A. galli* eggs are able to survive in the environment for more than 3 years (Permin, unpublished data). However, the time period that *S. enterica* could survive, either on the surface of the eggs or internalised, remains to be investigated.

Considering the present findings, rotation of birds should be investigated in organic and free-range table egg production, to establish criteria for minimising the risk of persistent *A. galli* infections and thus reduce the risk of persistence of *S. enterica* and other pathogens on these farms. Permin et al. (1998) have looked at the effect of stocking rates on the onset and spread of *A. galli* infections. An increased number of animals per unit area may lead to larger amounts of deposited faecal material and possibly higher infectivity per unit area. However, the development of eggs into infective stages and the survival of eggs in the environment presumably also affect the level of infectivity. Adequate cleaning and disinfection must be performed to minimise any chance of further dissemination of either the nematode and/or *Salmonella*, but even then survival of the parasite eggs is possible. If *Salmonella* can be internalised within the eggs, it may be protected from such disinfection procedures and may still present a threat of further contamination and spread. The possible contribution of other parasites prevalent in flocks kept under free-range conditions also needs to be addressed.

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## References

Ackert JE (1931) The morphology and life history of the fowl nematode *Ascaridia lineata* (Schneider). Parasitology 23: 360–379

Adedeji SO, Ogunba EO, Dipeolu OO (1989) Synergistic effect of migrating *Ascaris* larvae and *Escherichia coli* in piglets. J Helminthol 63: 19–24

Anderson RC (1992) Nematode parasites of vertebrates. Their development and transmission. CAB International, Cambridge University Press, Cambridge

Araujo P, Bressan CRV (1977) Consideration sur la deuxieme mue des larves *d'Ascaridia galli*. Ann Parasitol 5: 531–537

Barrow PA, Simpson JM, Lovell MA (1988) Intestinal colonisation in the chicken by food poisoning *Salmonella* serotypes: microbial characteristics associated with faecal excretion. Avian Pathol 17: 571–588

Bottjer KP, Hirsh DC, Slonka GF (1978) *Nematospiroides dubius* as a vector for *Salmonella typhimurium*. Am J Vet Res 39: 151–153

Brown HW, Perna VP (1958) An overwhelming *Strongyloides* infection. JAMA 168: 1648–1651

Calnek BW, Barnes HJ, Beard CW, Reid WM, Yoder HW Jr (1997) Diseases of poultry, 10th edn. Iowa State University Press, Des Moines

Cruthers LR, Weise RW, Hansen MF (1974) Topography of *Ascaridia galli* eggs exposed to low temperatures or cryoprotectants as shown by scanning electron microscopy. J Parasitol 4: 632–635

Eriksen L (1981) Host–parasite relations in *Ascaris suum* infection in pigs and mice. PhD thesis, Royal Veterinary and Agricultural University, Copenhagen

Hansen MF, Terhaar CJ, Turner DS (1956) Importance of the egg shell of *Ascaridia galli* to the infectivity of its larvae. J Parasitol 42: 122–125

Jensen HE, Schønheyder H (1989) Immunofluorescence staining of hyphae in the histopathological diagnosis of mycoses in cattle. J Med Vet Mycol 27: 33–44

Johnson J, Reid WM (1973) *Ascaridia galli* (Nematoda): development and survival in gnotobiotic chickens. Exp Parasitol 33: 95–99

Kates KC, Colglazier ML (1970) Differential morphology of adult *Ascaridia galli* (Schrank 1788) and *Ascaridia dissimilis* (Perez Vigueras 1931). Proc Helminthol Soc Wash 37: 80–84

Lund EE (1958) Growth and development of *Heterakis gallinae* in turkeys and chickens infected with *Histomonas meleagridis*. J Parasitol 44: 297–301

Lund EE, Chute AM (1973) Means of acquisition of *Histomonas meleagridis* by eggs of *Heterakis gallinarum*. Parasitology 66: 335–342

Lund EE, Wehr EE, Ellis DS (1966) Earthworm transmission of *Heterakis* and *Histomonas* to turkeys and chickens. J Parasitol 52: 899–902

Murray RGE, Doetsch RN, Robinow CF (1994) Determinative and cytological light microscopy. In: Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society of Microbiology, Washington, p. 38

Nalin DR, McLaughlin J (1976) Colonization of *Ascaris lumbricoides* by *V. cholerae*. J Parasitol 62: 839–841

Neves J, Marinho RP, Martins NR (1969) Prolonged septicaemic salmonellosis: treatment of intercurrent schistosomiasis with nitridazole. Trans R Soc Trop Med Hyg 63: 79–84

Niimi D (1937) Studies on blackhead. II. Mode of infection. [English summary, pp 23–26] J Jap Soc Vet Sci 16: 183–239

Nordentoft S, Christensen H, Wegener HC (1997) Evaluation of a fluorescence-labelled oligonucleotide probe targeting 23S rRNA for in situ detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears. J Clin Micro 35: 2642–2648

O'Brien ADP (1988) *Salmonella enteritidis* infection in broiler chickens. Vet Rec 122: 214

Okulewicz A, Zlotorzycka J (1985) Connection between *Ascaridia galli* and the bacterial flora in the intestine of hens. Angew Parasitol 26: 151–155

Ottens H, Dickerson G (1972) Studies on the effects of bacteria on experimental schistosome infections in animals. Trans R Soc Trop Med Hyg 66: 85–107

Pantelouris EM (1965) The common liver fluke. (International series of monographs on pure and applied biology/zoology division, vol 21) Pergamon Press, Oxford

Permin A (1997) Helminths and helminthosis in poultry with special emphasis on *Ascaridia galli* in chickens. PhD thesis, Royal Veterinary and Agricultural University, Copenhagen

Permin A, Pearman M, Nansen P, Bisgaard M, Frandsen F (1997) An investigation on different media for embryonation of *Ascaridia galli* eggs. Helminthology 34: 75–79

Permin A, Nansen P, Bisgaard M, Frandsen F, Pearman M (1998) Investigations on the infection and transmission of *Ascaridia galli* in free range chickens kept at different stocking rates. Avian Pathol 27: 382–389

Permin A, Bisgaard M, Frandsen F, Pearman M, Nansen P, Kold J (1999) The prevalence of gastrointestinal helminths in different poultry production systems. Br Poult Sci 40: 439–443

Pospischil A, Wood RL, Anderson TD (1990) Peroxidase-antiperoxidase and immunogold labelling of *Salmonella typhimurium* and *Salmonella choleraesuis* var *kunzendorf* in tissues of experimentally infected swine. Am J Vet Res 51: 619

Soulsby EJL (1982) Helminths, arthropods and protozoa of domestic animals, 7th edn. Baillière Tindall, London

Todd AC, Crowdus DH (1952) Factors influencing growth of a parasitic nematode. Trans Am Microbiol Soc 2: 102–108

Tyzzer EE (1926) *Heterakis vesicularis*, Froelich 1791: a vector of an infectious disease. Proc Soc Exp Med 23: 708–709

Tyzzer EE (1932) Problems and observations concerning the transmission of blackhead infection in turkeys. Proc Am Phil Soc 71: 407–410

Walker-Smith JA, McMillian B, Midleton AW (1969) Strongyloidases causing small-bowel obstruction in an aboriginal infant. Med J Aust 56: 1263–1265

Wilson S, Thompson AE (1964) A fatal case of strongyloidiases. J Pathol 87: 169–176