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Experimental infection of laying hens with *Serpulina intermedia* causes reduced egg production and increased faecal water content

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Serpulina intermedia strain HB60, isolated from an Australian hen with diarrhoea, was used to infect 10 individually caged 14-week-old laying hens. Another 10 birds were sham inoculated with sterile broth. Birds were kept for 16 weeks, and faecal water content, egg production and body weights recorded. Strain HB60 was isolated from the faeces of nine of the infected birds at irregular intervals throughout the experiment, and from their caeca at slaughter. Infected birds tended to be lighter and their faeces, on average, were significantly wetter (by 2.85%; $P < 0.002$) than those of the controls. Significant reductions in mean number of eggs laid (1.4/week; $P < 0.002$) and mean egg weights (1.16 g; $P < 0.05$) were recorded in infected birds. Colonization did not induce any characteristic pathological changes. *S. intermedia* is potentially an economically significant cause of reduced egg production, and wet faeces in layer and broiler breeder flocks.

Introduction

Anaerobic intestinal spirochaetes of the genus *Serpulina* colonize the large intestines of a variety of animal species. Three species are considered pathogens of the pig: the strongly haemolytic *S. hyodysenteriae* is the aetiological agent of swine dysentery (Stanton, 1992), whilst the weakly haemolytic *S. pilosicoli* and *S. intermedia*, respectively, cause porcine intestinal spirochaetosis (Trott *et al.*, 1996) and a more ill-defined colonic infection referred to as 'spirochaetal colitis' (Hampson & Trott, 1995; Stanton *et al.*, 1997).

Interest in the role of intestinal spirochaetes as pathogens of chickens has increased in recent years, following the publication of a series of reports implicating these bacteria as a cause of diarrhoea, reduced egg production and/or faecal staining of eggshells in layer and broiler breeder flocks in the Netherlands (Davelaar *et al.*, 1986; Dwars *et al.*, 1989, 1990, 1992b, 1993; Smit *et al.*, 1998), England (Griffiths *et al.*, 1987), the USA (Swayne *et al.*, 1992; Trampel *et al.*, 1994), and Australia (McLaren *et al.*, 1996). The bacteria involved were all weakly haemolytic, but otherwise were poorly characterized, and it was not

clear whether the various reports even referred to spirochaetes of the same species. Although some of these isolates had been used in experimental reproduction of disease in both Europe and the United States of America (USA), again it was not known how these strains were related to each other, if at all.

This situation was clarified by a study in which a variety of chicken strains from the various studies were analysed using multilocus enzyme electrophoresis (MLEE) (McLaren *et al.*, 1997). Six different genetic groups of chicken spirochaetes were identified, each equated with a separate species in the genus *Serpulina*. Isolates that had previously been used to induce disease in broiler chicks and/or layers in Europe and the USA were shown to belong to three separate species. It transpired that strain 1380, used in experimental infection studies in layers and broilers in the Netherlands (Dwars *et al.*, 1992a,b, 1993), was a strain of *S. intermedia* (called '*S. intermedius*' by McLaren *et al.*, 1997). Similarly, it was shown that an *S. intermedia* strain had been implicated in the production problems described in England (Griffiths *et al.*, 1987). In contrast, in the USA workers had

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been dealing either with the recently-named *S. alvinipulli* (Swayne *et al.*, 1995; Stanton *et al.*, 1998) or with *S. pilosicoli* (Trampel *et al.*, 1994). In Western Australia, where 35% of layer flocks and 53.3% of broiler breeder flocks were found to be infected with intestinal spirochaetes (McLaren *et al.*, 1996), 29% of the isolates were shown to be *S. intermedia* (McLaren *et al.*, 1997). A single *S. pilosicoli* isolate was recorded from a flock in Queensland, and no isolates of *S. alvinipulli* were recovered (McLaren *et al.*, 1997).

In view of the high prevalence of *S. intermedia* infections in layer and broiler breeder flocks in Australia, and the fact that a strain of this species has been used experimentally to induce disease in chickens in Europe, this study was undertaken to investigate the pathogenic potential of a representative Australian strain of *S. intermedia* in laying hens.

Materials and Methods

Experimental birds

Twenty commercial layer hens were purchased at 14 weeks of age, randomly divided into two groups of 10 birds and housed in individual cages in two separate rooms of an isolation animal house. Feed and equipment for the two rooms were kept separate, and personnel moving between the rooms changed into fresh protective clothing to avoid possible transmission of infection between rooms. The birds were fed a commercial layer diet and fresh water *ad libitum* throughout the experiment. The birds were subjected to natural lighting, which at the time of the experiment involved cycles of approximately 12 h of full daylight and 12 h of darkness.

Experimental infection and monitoring of birds

Western Australian *Serpulina intermedia* strain HB60, originally recovered from a laying hen with diarrhoea (McLaren *et al.*, 1996, 1997), was obtained from the collection held at the Reference Laboratory for Intestinal Spirochaetes at Murdoch University. This strain was selected because it was more strongly haemolytic than most strains of *S. intermedia* and, hence, its presence was easier to monitor on isolation plates. The frozen strain was thawed and grown to mid-log phase in Kunkle's pre-reduced anaerobic broth medium (Kunkle *et al.*, 1986), to a density of approximately 10^8 cells/ml as assessed by direct counts in a haemocytometer chamber viewed through a phase contrast microscope. Birds in one group were then each inoculated with 5 ml of active broth culture (5×10^8 cells) by crop tube daily for three days. The other group of birds received the same amount of sterile broth culture over the same period. The birds were checked twice daily for signs of ill-health. Prior to inoculation all birds were weighed and faecal swabs taken, and this was repeated at 2-week intervals post-infection (p.i.) over 16 weeks. Faeces were cultured for spirochaetes on Trypticase soy agar plates supplemented with 5% defibrinated ovine blood, 400 µg/ml spectinomycin, and 25 µg each of colistin and vancomycin, and incubated at 37°C for 7 days under an atmosphere of 94% NO₂ and 6% CO₂ (McLaren *et al.*, 1996). Selected weakly haemolytic isolates were confirmed as *S. intermedia* by testing for indole production from growth in Kunkle's broth, and a subset of isolates were examined by MLEE, as previously described (McLaren *et al.*, 1997). Percentage faecal dry matter content was determined by drying weighed faecal samples to constant weight. Eggs were collected and weighed daily, once laying commenced regularly at 22 weeks of age.

When the birds reached 30 weeks of age they were killed by an intravenous injection of barbiturate. Sections from the mid-portion of the wall of one caecum were then immediately taken for histopathol-

ogy. They were placed into Bouin's fixative for 4 h before being washed three times in 50% ethanol and being processed conventionally. Sections were cut at 4 µm and stained with haematoxylin and eosin, and with Warthin–Starry silver stain. Material for scanning electron microscopy was placed into chilled half strength Karnovsky's fixative for 24 h and then post-fixed in 1% aqueous osmium tetroxide for 1 h at 4°C. These were dehydrated, critical point dried and sputter-coated with gold before viewing under a Philips XL scanning electron microscope. The other caecum was opened and the contents plated directly to the selective spirochaete medium.

Analysis

Results for faecal dry matter for the birds in the two groups were compared over the whole experimental period using one-way analysis of variance to evaluate the significance of group differences, with differences in weekly results analysed using least significant difference values. Results for egg production and egg weights were only compared over the period 10 to 16 weeks p.i., once the birds were in full lay. Weekly egg numbers over the whole experimental period were compared using Student's *t*-test, whilst one-way analysis of variance and least significant difference was used for egg weights.

Results

Colonization

No birds were colonized with intestinal spirochaetes prior to the start of the experiment and none of the control birds was colonized at any time. Spirochaetes were isolated from nine of the experimentally-infected birds at irregular periods p.i., and were isolated from the caeca of all nine at slaughter. All isolates tested were confirmed to have the same electrophoretic type as HB60 by MLEE. One bird in the infected group was not colonized at any time and so results for it were excluded from the analysis. One of the control birds failed to commence lay and so also was excluded from the analysis.

The average weight of the nine birds in the infected group was consistently less than that of the controls from week 2 p.i. onwards (Table 1), but overall this difference failed to reach significance.

Faecal dry matter

Group mean values for faecal dry matter content are presented in Table 2. Dry matter content did not differ significantly in the two groups at the outset of the experiment (week 0), but did decrease in both groups by week 2. Subsequently, the infected birds were observed to have wetter and less well-formed faeces than the control birds, and the dry matter content over the experimental period was reduced from a group mean of 22.73% in control birds to 19.88% in infected birds. This difference between groups over the period 2 to 16 weeks (mean 2.85% overall) was statistically significant ($P < 0.002$).

Table 1. Group mean body weight (\pm standard deviation) in grams of adult layers at two-weekly intervals following infection with *S. intermedia* strain HB60 at 14 weeks of age

Week	Control	Infected
0	1141.4 \pm 121.1	1214.7 \pm 62.8
2	1403.3 \pm 112.0	1349.1 \pm 80.7
4	1497.4 \pm 137.3	1431.3 \pm 92.2
6	1625.7 \pm 153.4	1547.1 \pm 103.0
8	1690.5 \pm 192.6	1597.0 \pm 100.2
10	1701.7 \pm 247.5	1605.5 \pm 127.0
12	1650.2 \pm 255.6	1546.1 \pm 140.8
14	1709.2 \pm 205.6	1514.0 \pm 177.3
16	1701.7 \pm 229.9	1553.0 \pm 154.3

Table 2. Group mean percentage faecal dry weights (\pm standard deviation) and significance, using least significant difference from one-way analysis of variance, in adult layers in weeks after infection with *S. intermedia* strain HB60

Weeks	Uninfected	Infected	Significance
Week 0	27.13 \pm 3.94	28.53 \pm 5.50	NS
Week 2	23.80 \pm 2.03	20.82 \pm 3.93	*
Week 4	24.98 \pm 2.53	20.61 \pm 2.85	**
Week 6	20.58 \pm 3.32	18.72 \pm 2.23	NS
Week 8	24.19 \pm 3.23	19.71 \pm 4.04	*
Week 10	21.36 \pm 0.99	21.03 \pm 2.27	NS
Week 12	22.43 \pm 3.10	17.57 \pm 4.79	*
Week 14	22.28 \pm 3.45	21.02 \pm 4.43	NS
Week 16	22.19 \pm 2.10	19.55 \pm 4.68	NS

NS = not significant; * $P < 0.05$; ** $P < 0.01$.

Egg production

Group mean weekly results for egg production and egg weights are presented in Table 3. Over the 7-week period from when regular lay had commenced (10 to 16 weeks p.i.) the nine control birds produced an average of 5.3 eggs per week compared to 3.9 per week for the infected birds. This difference (1.4 eggs/week) was statistically significant ($P < 0.002$). Over the period the average weight of the eggs produced by the control birds was 45.96 g compared to 44.80 g for the infected birds, and again this difference of 1.16 g/egg was statistically significant ($P < 0.05$).

Post-mortem findings

At post-mortem examination the infected birds were observed to have less abdominal fat, and their caeca contained more gas and the contents were more fluid than those of the control birds. No obvious consistent gross or histological lesions were found in the caeca of birds in either group. Scanning electron microscopy revealed large numbers of spirochaetes overlaying and loosely associ-

ated with the caecal epithelium in all nine birds from the infected group.

Discussion

Natural infection with *S. intermedia* has been reported in laying hens and broiler breeders, and appears to occur commonly in both Europe and Australia. It has not been reported from North America. Experimental infection of adult birds with Dutch *S. intermedia* strain 1380 resulted in increased faecal water content, reduced egg production, and poor performance in broilers hatched from the infected parents (Dwars *et al.*, 1989; 1992b; 1993). The same strain also caused depressed growth and increased faecal fat in experimentally infected broilers (Dwars *et al.*, 1992a). The current results using an Australian strain of *S. intermedia* in a small group of commercial birds approaching lay were consistent with the findings in adult birds in the Netherlands.

One inoculated bird was apparently resistant to colonization, but the others became persistently colonized over a 16-week-period and scanning electron microscopy revealed large numbers of organisms overlying their caecal epithelium. The persistent nature of the experimental infection was consistent with reports of field cases in the Netherlands (Daveleer *et al.*, 1986). The growth rates of the experimentally-infected birds appeared depressed, but probably as a result of the small group size, differences were not significant.

Faecal dry matter content in birds in both groups was reduced considerably between weeks 0 and 2 (Table 2), although no obvious difference in faecal consistency was detected visually. It is assumed that this increase in faecal water content was associated with factors such introduction to a new environment and a change in diet. Subsequently, colonization by *S. intermedia* was associated with a significant and persistent increase in faecal moisture content (mean increase of 2.9%) and this was sufficient to make the faeces appear much more fluid. Under commercial situations loose faeces such as those produced here can result in serious problems, including faecal staining of egg shells, mechanical problems with removal of excreta, increased odour and attraction of flies. Weekly production in these infected birds was reduced by an average of 1.4 eggs/bird over a 7-week period, with the eggs produced being on average 1.16 g lighter than those from uninfected birds held under the same conditions. If these losses extended over the whole cycle of production, there is little doubt that they would have a serious economic impact in an infected flock.

Interestingly, no gross or histological changes were found in the colonized caeca, although the infected birds were in poorer body condition than the control birds. The lack of pathological changes may have been because of the chronic nature of the

Table 3. Group mean weekly egg production and mean egg weights in grams in infected and uninfected birds at weeks 10 to 16 post-infection (p.i.) with *S. intermedia* strain HB60, and significance from least significant significance in one-way analysis of variance

Week (p.i.)	Mean egg production (eggs/hen/week)		Mean egg weights (g ± SD)		Significance
	Controls	Infected	Controls	Infected	
10	5.4	4.1	41.44 ± 2.96	42.82 ± 3.85	NS
11	4.3	3.4	45.18 ± 5.21	43.65 ± 3.38	NS
12	5.4	4.2	46.33 ± 4.81	44.39 ± 3.54	*
13	5.1	4.1	45.42 ± 5.76	44.89 ± 3.62	NS
14	4.7	4.1	46.46 ± 3.00	44.97 ± 3.28	*
15	5.8	3.2	48.08 ± 4.40	46.24 ± 3.25	NS
16	6.3	4.1	48.80 ± 4.23	46.67 ± 2.78	**

NS = not significant; * $P < 0.05$; ** $P < 0.01$.

colonization, and more acute lesions may have been present earlier. The lack of obvious change in the caeca, other than their fluid content, means that it may not be possible to diagnose *S. intermedia* infection on pathological grounds. Diagnosis therefore is best based on appropriate microbiological examination of birds that are showing suggestive clinical signs. The anaerobic nature and fastidious growth requirements of the spirochaete unfortunately also make routine microbiological diagnosis relatively difficult.

This study has confirmed the pathogenic potential of *S. intermedia* strains in laying birds, and has demonstrated that Australian strains are as likely to be problematic as are strains present in Europe. In view of the widespread distribution of *S. intermedia* in flocks in Europe and Australia, this organism is almost certainly causing serious economic losses. Further experimental studies using more birds, held over a longer period, are now required to confirm and extend these findings. Improved means to diagnose and control *S. intermedia* infections in the field are also urgently required.

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RÉSUMÉ

Infection expérimentale de poules pondeuses par *Serpulina intermedia* entraînant une diminution de la production d'oeufs et une augmentation de la teneur en eau des matières fécales

La souche HB60 de *Serpulina intermedia* a été isolée en Australie à partir d'une poule pondeuse présentant de la diarrhée et a été utilisée pour infecter 10 futures pondeuses âgées de 14 semaines, maintenues en cages individuelles. Dix autres oiseaux ont été inoculés avec du bouillon stérile. Les oiseaux ont été gardés en observation durant 16 semaines. La teneur en eau des matières fécales, la production d'oeufs et le poids des corps ont été enregistrés. La souche HB60 a été isolée à partir de fèces de neuf des oiseaux infectés à intervalles réguliers au cours de l'expérimentation et à partir de leurs caeca lors de l'abattage. Les oiseaux infectés ont eu tendance à être moins lourds et leurs fèces avaient en moyenne une teneur en eau significativement plus importante que ceux des témoins (près de 2,85%, $P < 0,002$). Des réductions significatives du nombre moyen d'oeufs pondus (1,4/semaine; $P < 0,002$) et du poids moyen des oeufs (1,16 g; $P < 0,05$) ont été enregistrées chez les oiseaux infectés. La colonisation n'a entraîné aucun trouble pathologique. *S. intermedia* paraît être un agent causal responsable sur le plan économique, en réduisant la production d'oeufs et en augmentant la teneur en eau des fèces chez les poules pondeuses et les reproducteurs.

ZUSAMENFASSUNG

Die experimentelle Infektion von Legehennen mit *Serpulina intermedia* verursacht eine verminderte Eierproduktion und einen erhöhten Wassergehalt des Kots

Der aus einer australischen Henne mit Durchfall isolierte *Serpulina intermedia*-Stamm HB60 wurde für die Infektion von 10 einzeln in Käfigen gehaltenen 14 Wochen alten Legehennen verwendet. Weitere 10 Hennen wurden mit steriler Nährbouillon scheininfiziert. Die Tiere wurden 16 Wochen lang gehalten, und der Wassergehalt des Kots, die Eierproduktion und die Körpergewichte wurden dokumentiert. Der

Stamm HB60 wurde von neun der infizierten Tiere das ganze Experiment hindurch in unregelmäßigen Abständen aus dem Kot sowie bei der Schlachtung aus ihren Blinddärmen isoliert. Die infizierten Tiere tendierten zu geringerem Körpergewicht, und ihr Kot war im Durchschnitt signifikant feuchter (um 2,85%; $P < 0,002$) als der von den Kontrollen. Signifikante Verminderungen der mittleren Anzahl gelegter Eier (1,4/Woche; $P < 0,002$) und der mittleren Eigewichte (1,16 g; $P < 0,05$) wurden bei den infizierten Tieren registriert. Die Keimbeseidlung verursachte keinerlei charakteristische pathologische Veränderungen. *S. intermedia* ist möglicherweise eine wirtschaftlich wichtige Ursache von verminderter Eierproduktion und nassem Kot in Legehennen- und Broiler-Elterntierherden.

RESUMEN

La infección experimental de gallinas de puesta con *Serpulina intermedia* produce una disminución de la puesta y un incremento en el contenido acuoso fecal

Se utilizó la cepa HB60 de *Serpulina intermedia*, procedente de una gallina australiana con un cuadro diarreico, para infectar diez gallinas de puesta de 14 días de vida, localizadas en jaulas individualizadas. Otras 10 aves fueron inoculadas con un caldo estéril. Se hizo un seguimiento de 16 semanas, registrándose el contenido acuoso de las heces, la producción de huevos y el peso corporal. La cepa HB60 se aisló a partir de las heces de nueve de los animales infectados en intervalos irregulares, a lo largo del experimento y a partir de sus ciegos, en el matadero. Las aves infectadas tendían a presentar heces de baja consistencia, que tenían una media de humedad significativamente mayor (2.85%; $P < 0.002$) que la de los controles. En las aves infectadas se registraron reducciones significativas en la media de los huevos puestos (1.4/semana; $P < 0.002$) y en el peso medio de éstos (1.16 g; $P < 0.05$). La colonización no daba lugar a cambios patológicos significativos. *S. intermedia* es potencialmente una causa económicamente significativa de reducción de la puesta y humedad en las heces en ponedoras y reproductores.