Epidemiology of *Yersinia pseudotuberculosis* and *Y. enterocolitica* Infections in Sheep in Australia

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Infections with *Yersinia pseudotuberculosis* serotype III and *Y. enterocolitica* serotype O2,3 were found to be common in Australian sheep flocks. Transmission of *Y. pseudotuberculosis* occurred in late winter and early spring, while *Y. enterocolitica* transmission occurred from midwinter to early summer. Excretion of *Y. pseudotuberculosis* was limited to the winter and spring period and was particularly common in 1- and 2-year-old sheep. Infection persisted for up to 14 weeks. *Y. pseudotuberculosis* infection did not confer immunity to natural infection with *Y. enterocolitica*. *Y. enterocolitica* excretion occurred year-round, with the greatest prevalence being in summer and autumn. Infection persisted for up to 29 weeks. Sheep less than 1 year old were most commonly infected with *Y. enterocolitica*. Infection with either *Y. pseudotuberculosis* or *Y. enterocolitica* was rare in aged sheep. Restriction endonuclease analysis of *Y. pseudotuberculosis* serotype III from sheep, cattle, deer, and pigs showed that the bacterial isolates were genetically indistinguishable. Similarly, *Y. enterocolitica* isolates from sheep were indistinguishable from those isolated from goats and cattle.

The Gippsland region of Victoria, Australia, is a major sheep- and cattle-raising area. Livestock graze on pasture for 12 months of the year, and housing is not necessary. Winters are mild, and summers are warm, with most of the rain falling in winter and spring.

Sheep in the study area are raised for both meat and wool production. Merino and British breeds and their crosses are all present. The number of sheep on a farm varies, but a range of several hundred to several thousand animals is usual. Cattle are often grazed with sheep. Sheep on farms are normally allocated into groups based on gender and age class and are managed as separate flocks.

*Yersinia pseudotuberculosis* serotypes I, II, and III are known to be enteropathogens of a range of ungulate species in this area, including cattle (11), deer (5), and sheep, goats, and pigs (12). Characteristic *Yersinia enterocolitica* serotype O2,3 strains that most closely resemble biotype 5 of Ewing (1) are a common enteropathogen of Gippsland sheep and goats (13) and, on rare occasions, of cattle (our unpublished data). Avirulent strains of *Yersinia* spp. have also been isolated in the study area (our unpublished data). *Yersinia* spp. have been recognized as pathogens of a range of other animal species, including humans. Considerable effort has been devoted to the investigation of possible sources of human infection (1), but few epidemiological investigations have been undertaken within a host species.

While virulent strains of both *Y. pseudotuberculosis* serotype III and *Y. enterocolitica* serotype O2,3 produce typical intestinal microabscesses in sheep (12, 13), avirulent strains do not (our unpublished data).

This study was undertaken to determine the prevalence of virulent *Yersinia* spp. in commercial sheep flocks in the Gippsland region of Victoria and to investigate epidemiological aspects of the infection by monitoring *Yersinia* transmission in an experimental flock. Restriction endonuclease analysis (REA) of chromosomal DNA from field isolates of *Y. enterocolitica* and *Y. pseudotuberculosis* was performed in an attempt to detect genetic differences that might relate to host specificity.

MATERIALS AND METHODS

**Field survey.** This study was carried out from July 1988 to December 1989 as an adjunct to a program to monitor gastrointestinal helminth burdens in commercial sheep flocks. Fecal samples were collected from 10 sheep in each of 449 flocks and either hand delivered to the laboratory or sent by mail. Of the 10 fecal samples, 4 were randomly selected and cultured for *Yersinia* spp. A brief flock history, including information on the breeds and ages of the sheep and some clinical details, was usually provided.

A flock was considered infected when one or more colonies of virulent *Y. pseudotuberculosis* or *Y. enterocolitica* were isolated from at least one of the four sheep tested.

**Monitoring of the experimental flock.** A flock of 20 7-year-old crossbred ewes and their offspring was maintained at the laboratory. Although this flock was small compared with commercial flocks in the area, management practices, including stocking rates, were similar to those of flocks in the field survey.

Rectal swabs collected from the laboratory flock were cultured for *Yersinia* spp. at weekly intervals between July 1989 and April 1991. A sheep was considered infected when virulent *Y. pseudotuberculosis* or *Y. enterocolitica* was isolated from a rectal swab.

Lambs were born between late June and early November 1989 and between mid-July and early August 1990 and were weaned at 8 to 12 weeks by separating them from their mothers for a period of 2 to 3 weeks.

The flock was grazed in paddocks that had held sheep, goats, and cattle in previous years. Ewes were culled from the flock if disabilities or diseases developed. A number of lambs born in 1989 were removed from the flock prior to the 1990 lambing because of a lack of pasture. Three merino sheep that were excreting *Y. enterocolitica* were introduced to the flock in February 1990.

**Microbiology.** A selective culture medium, CIN agar (Ox-
oid Australia Pty Ltd., West Heidelberg, Victoria, Australia), was used for the isolation of Yersinia spp. from feces and rectal swabs. Specimens were inoculated directly onto culture plates which were then incubated for 40 h at 30°C (11). Cold enrichment of samples and the use of other agars selective for Yersinia spp. were deliberately avoided. It was considered desirable to employ a relatively insensitive culture technique which would minimize the detection of ingested bacteria passing passively through the gastrointestinal tract without causing infection.

Suspect Yersinia colonies growing on CIN agar were subcultured and screened for urease production. One colony of each morphological type was tested. Urease-positive isolates were further tested for their ability to produce acid from the carbohydrates cellobiose, melibiose, rhamnose, and sucrose (11).

The potential virulence of Yersinia isolates was investigated by testing for calcium dependence on MOX agar (10). Virulent, calcium-dependent isolates were serotyped with antisera prepared in rabbits. Virulent Y. enterocolitica isolates were biotyped for testing the production of DNase, indole, and lipase and for the ability to reduce nitrate to nitrite and to ferment trehalose and xylose (1).

REA of chromosomal DNA. REA of chromosomal DNA was performed with 80 isolates of virulent Y. enterocolitica and 41 isolates of virulent Y. pseudotuberculosis obtained either during this study or from samples submitted to the laboratory for disease diagnosis. Isolates of Y. enterocolitica from 74 sheep, 4 cattle, and 2 goats were tested. Isolates of Y. pseudotuberculosis were type I isolated from 2 cattle, 2 deer, 1 sheep, and 1 goat, while serotype III isolates were obtained from 19 sheep, 12 cattle, 3 deer, and 1 pig.

Chromosomal DNA was prepared by using a modification of a previously described method (8). Yersinia spp. were harvested from 2-day-old cultures grown aerobically on blood agar plates incubated at 30°C. Colonies were scraped from the agar and suspended in 1 ml of lysing buffer (100 mM Tris-HCl, 100 mM EDTA [pH 8.0]). Lysozyme was added (100 μl at 3 mg/ml), and the cells were incubated at 37°C for 15 min. This was followed by the addition of 100 μl each of pronase (10 mg/ml) and 10% sodium lauryl sulfate and further incubation at 60°C overnight. Sodium percarbonate (350 μl at 5 M) was added, and incubation continued at 60°C for 60 min. STE buffer (4 ml of 100 mM NaCl–50 mM Tris-HCl–1 mM EDTA [pH 7.5]) was added, and the cell lysate was extracted twice with phenol-chloroform-isooamyl alcohol (25:24:1). The aqueous DNA solution was then precipitated by the addition of a 1/20 volume of 5 M NaCl and 2 volumes of ethanol, washed with 70% ethanol, quickly rinsed with distilled water, and dissolved in 500 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]). Approximately 1 μg of DNA was digested with HhaI restriction endonuclease according to the manufacturer’s (Molecular Biology Division, Pharmacia, Uppsala, Sweden) instructions. The DNA digests were separated electrophoretically on 0.7% agarose gels (20 by 20 cm) for 16 h at 40 V. Gels were stained for 60 min in 0.2 μg of ethidium bromide per ml, rinsed briefly in distilled water, and photographed under shortwave UV transillumination through Kodak 9 and 23A gelatin filters.

**RESULTS**

Field survey. Merino- and British-breed sheep and their crosses were all represented in the field survey. Y. pseudotuberculosis was isolated from one or more sheep in 21 (5%) of 449 flocks (Table 1). When a sheep first became infected, numerous typical bacterial colonies were usually present on CIN agar; however, with time the number of colonies diminished until excretion was no longer detected. All Y. pseudotuberculosis isolates were calcium dependent and were therefore considered virulent. Of the Y. pseudotuberculosis isolates, 1 was serotype I and the remaining 20 were serotype III. Virulent Y. enterocolitica was isolated from one or more sheep in 78 (17%) of 449 flocks (Table 1). The number of colonies grown from samples ranged from as few as one to many hundreds. Virulent Y. enterocolitica isolates were all serotype O2,3, produced DNase, fermented sucrose, did not produce indole or lipase, failed to reduce nitrates to nitrite, and varied in their abilities to ferment trehalose and xylose. Occasional colonies of avirulent Yersinia spp. were grown from fecal samples tested during the winter and spring months, but once identified, these bacteria were not considered further and are not included in Tables 1 and 2.

Y. pseudotuberculosis infection occurred seasonally, while infections with virulent Y. enterocolitica were detected throughout the year (Table 1). Y. pseudotuberculosis infection occurred most commonly in 1- and 2-year-old sheep, while infection with virulent Y. enterocolitica was most common in sheep less than 1 year old (Table 2).

**Monitoring of the experimental flock.** Occasional colonies of avirulent Yersinia spp. were cultured from both ewes and

<table>
<thead>
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<th>Mo</th>
<th>Y. enterocolitica (%)</th>
<th>Y. pseudotuberculosis (%)</th>
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<tbody>
<tr>
<td>January</td>
<td>6/26 (23)</td>
<td>0/26 (0)</td>
</tr>
<tr>
<td>February</td>
<td>11/38 (29)</td>
<td>0/38 (0)</td>
</tr>
<tr>
<td>March</td>
<td>9/33 (27)</td>
<td>0/33 (0)</td>
</tr>
<tr>
<td>April</td>
<td>2/22 (9)</td>
<td>0/22 (0)</td>
</tr>
<tr>
<td>May</td>
<td>7/39 (18)</td>
<td>0/39 (0)</td>
</tr>
<tr>
<td>June</td>
<td>4/42 (12)</td>
<td>1/42 (2)</td>
</tr>
<tr>
<td>July</td>
<td>4/32 (13)</td>
<td>4/32 (13)</td>
</tr>
<tr>
<td>August</td>
<td>5/55 (9)</td>
<td>9/55 (16)</td>
</tr>
<tr>
<td>September</td>
<td>4/40 (10)</td>
<td>4/40 (10)</td>
</tr>
<tr>
<td>October</td>
<td>6/36 (17)</td>
<td>3/36 (8)</td>
</tr>
<tr>
<td>November</td>
<td>10/42 (24)</td>
<td>0/42 (0)</td>
</tr>
<tr>
<td>December</td>
<td>10/44 (23)</td>
<td>0/44 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>78/449 (17)</td>
<td>21/449 (5)</td>
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</table>

* Number positive per number tested.

<table>
<thead>
<tr>
<th>Age(s) yr</th>
<th>Y. enterocolitica (%)</th>
<th>Y. pseudotuberculosis (%)</th>
</tr>
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<tbody>
<tr>
<td>&lt;1</td>
<td>58/162 (36)</td>
<td>3/162 (2)</td>
</tr>
<tr>
<td>1</td>
<td>9/81 (11)</td>
<td>15/81 (19)</td>
</tr>
<tr>
<td>2</td>
<td>2/46 (4)</td>
<td>3/46 (7)</td>
</tr>
<tr>
<td>3</td>
<td>1/49 (2)</td>
<td>0/49 (0)</td>
</tr>
<tr>
<td>4</td>
<td>2/51 (4)</td>
<td>0/51 (0)</td>
</tr>
<tr>
<td>5</td>
<td>0/19 (0)</td>
<td>0/19 (0)</td>
</tr>
<tr>
<td>6 and 7</td>
<td>2/21 (10)</td>
<td>0/21 (0)</td>
</tr>
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</table>

* Number testing positive per total number tested.
lams from May to December in both 1989 and 1990. As in the field survey, once identified they were not considered further.

Although no source of Y. pseudotuberculosis serotype III was known to be present in the flock, infection with this bacterium spread among the 2- to 4-month-old lambs between late August and mid-October 1989. Eleven of 12 lambs but none of 19 ewes became infected. None of the infected lambs showed clinical evidence of disease. Excretion of Y. pseudotuberculosis persisted for 1 to 14 weeks in individual lambs and had ceased by mid-January 1990. Three lambs born in early November 1989 did not become infected with this bacterium, although other lambs in the flock were still excreting Y. pseudotuberculosis.

Although the flock was exposed from February 1990 to sheep that were excreting virulent Y. enterocolitica, transmission of infection to the experimental flock did not occur until late July. All Y. enterocolitica transmission ceased by early December. Of 8 lambs approximately 14 months old born in 1989, 5 became infected, as did 2 of 14 lambs 2 to 6 months old. Two of three lambs that had been infected with Y. pseudotuberculosis in 1989 became infected with Y. enterocolitica in 1990. In comparison, three of five lambs that had not been infected with Y. pseudotuberculosis in 1989 were infected with Y. enterocolitica in 1990. None of the 10 remaining ewes became infected with Y. enterocolitica.

No clinical disease was noted in lambs infected with Y. enterocolitica. Excretion of Y. enterocolitica persisted for 3 to 16 weeks. The two sheep that had been infected with Y. pseudotuberculosis during 1989 excreted Y. enterocolitica for 5 and 8 weeks. Lambs not previously infected with Y. pseudotuberculosis excreted Y. enterocolitica for 3 to 16 weeks. One of the merino sheep introduced to the flock to initiate Y. enterocolitica infection excreted the bacterium for at least 29 weeks. Excretion of Y. enterocolitica could no longer be detected in the experimental flock beginning in late January 1991.

REA of chromosomal DNA. REA of the 80 virulent Y. enterocolitica isolates showed that they consisted of four genotypes, designated A, B, C, and D (Fig. 1). Of the 74 sheep isolates, 50 were genotype A, 11 were genotype B, 12 were genotype C, and 1 was genotype D. Three of four cattle isolates were genotype B, and the other was genotype C. Both goat isolates were genotype A.

REA of Y. pseudotuberculosis serotype I revealed two genotypes, designated 1A and 1B (Fig. 1). Isolates from sheep, cattle, and goats were all genotype 1A, while both isolates from deer were genotype 1B. The 35 isolates of Y. pseudotuberculosis serotype III were, for the most part, indistinguishable regardless of the animal species of origin and were designated genotype IIIA. The exception was a single sheep isolate designated genotype IIB which had an additional DNA band with a size of approximately 3 kb (Fig. 1).

DISCUSSION

Virulent Y. pseudotuberculosis serotype III and Y. enterocolitica were frequently isolated from sheep in the flock survey (Table 1), and it appears that most flocks in the study area are infected. The Yersinia prevalence demonstrated by the field survey may have been biased to some extent because flocks were selected for testing to determine the degree of helminth infestation. However, gastrointestinal helminths are present in all flocks in the study area and are recognized as the major cause of diarrhea in sheep, so that bias is probably minimal.

Both Y. pseudotuberculosis serotype III and Y. enterocolitica are known enteropathogens of sheep and are excreted in large numbers during the acute stage of infection (12, 13). Our finding that infections with these bacteria are common in sheep in the study area suggests that sheep are a maintenance host for both species. Although Y. pseudotuberculosis serotype I was occasionally isolated from sheep, it appears that sheep are not a major maintenance host for this bacterium. Avirulent Yersinia spp. were isolated in small numbers from occasional sheep of all age classes in winter and spring. It is likely that these isolates were not causing infection but were being passed through the intestinal tract with food. Infection with avirulent Yersinia spp. is not associated with intestinal microabscess formation (our unpublished data).

The age distribution of infection (Table 2) indicates that most animals acquire infection in the first 2 years of life. It has been previously reported that younger sheep, goats, pigs, deer, and cattle are more likely to contract Y. pseudotuberculosis and Y. enterocolitica infections than are older animals (5, 11-13). As expected, Y. enterocolitica infection was most common in sheep less than 1 year old. However, the finding that Y. pseudotuberculosis infection was most common in 1-year-old sheep (Table 2) was unexpected. Possible reasons for this difference may be subtle variation in the period when environmental conditions are optimal for transmission of these bacterial species, differences in the persistence of maternally derived immunity in lambs, or variation in the way infection is maintained within flocks in the field. Older sheep in both the field survey and experimental flocks were seldom infected with virulent Yersinia spp., possibly because of past infection resulting in immunity to further challenge. Nonvirulent Yersinia spp. were, however, frequently isolated from older sheep.

Monitoring of the experimental flock indicated that transmission of both Y. enterocolitica and Y. pseudotuberculosis appeared to be limited to the cool, damp weather occurring between July and early December. Fecal excretion of Y.
pseudotuberculosis serotype III was very seasonal, occurring only during winter, spring, and early summer in both the field survey flocks (Table 1) and the experimental flock. Numerous authors have reported this seasonality of infection by Yersinia spp. in a range of domestic and wild mammals, human beings, and birds (3, 7, 11, 12). Excretion of Y. enterocolitica by sheep in the experimental flock was also restricted to winter, spring, and early summer. In contrast, the field survey demonstrated that Y. enterocolitica was excreted in all months of the year, with a possible peak in prevalence from late spring to early autumn (Table 1). This lack of seasonality in sheep has been reported previously (13). The reasons for this different excretion pattern are unknown, but the observation that a merino sheep introduced to the experimental flock excreted Y. enterocolitica for at least 29 weeks, more than twice as long as any of the crossbred sheep in the same flock, suggests that some breeds or lines of sheep may carry this bacterium for extended periods after initial infection.

Since two of three lambs in the experimental flock that had been infected with Y. pseudotuberculosis in 1989 became infected with Y. enterocolitica in 1990, it does not appear that cross immunity is conferred. Our finding that some lambs exposed to Y. pseudotuberculosis and Y. enterocolitica did not become infected may reflect failure to ingest an infectious dose of organisms, persistence of protective colostral antibodies, or inherent resistance to infection. Genetically mediated resistance to Y. pseudotuberculosis infection in farmed deer has been recorded (6).

REA indicated that a single genotype of Y. pseudotuberculosis serotype III infected sheep, cattle, deer, and pigs, while serotype I strains were of two genotypes, A and B. Genotype A, isolated from sheep, was also shown to infect cattle and goats, while genotype B was isolated from deer only. Whether both genotypes are capable of infecting sheep and whether these genotypes correspond to the Y. pseudotuberculosis serotypes IA and IB of Thal (14) are not known.

The only known sources of the virulent Y. enterocolitica strains isolated in this study are sheep and goats and, on rare occasions, cattle. REA of isolates indicated that at least four genotypes exist. Although only two goat isolates were tested, they were of a genotype common to sheep. Further isolates from goats need to be tested to determine whether all four genotypes are shared by goats and sheep. Isolates of Y. enterocolitica from cattle were also genetically indistinguishable from those found in sheep. As Y. enterocolitica is rarely isolated from cattle in the study area, it appears likely that cattle are infected by contact with sheep or goats and that cattle are not important in the maintenance or transmission of this bacterium.

Published observations indicate that a similar range of Yersinia spp. occurs in livestock species in both New Zealand (4, 9) and Australia (5, 11–13). The epidemiological picture described in this study also appears likely to be common to both countries. Whether this holds true for the rest of the world remains largely unknown at present, although pigs do not appear to be significant carriers of Y. enterocolitica in Australia (2, 12), in contrast to the situation in Europe described previously (15).

Because of the geographic isolation and unique fauna of Australia and New Zealand, it is possible that Yersinia spp. were not present in either country until European settlement occurred in the late 1700s and early 1800s. These bacteria may have been unknowingly introduced by human carriers or in shipments of domestic livestock, particularly from Europe or southern Africa. The long sea voyage or, in more recent times, the lengthy quarantine period imposed by Australia and New Zealand against animal introductions from other areas may have restricted the introduction of Yersinia spp., perhaps maintaining a relatively impoverished flora and the apparently simple epidemiological picture described here.

This study is one of the first comprehensive epidemiologic investigations of Yersinia infection. An examination of other potential hosts, particularly birds, rodents, and grazing animals, may be of value in elucidating the wider perspectives of these infections and in developing disease control strategies.

ACKNOWLEDGMENT

Cathy McLellan ably assisted with the isolation and identification of Yersinia spp.

REFERENCES


