Presence of *Mycobacterium avium* subspecies *paratuberculosis* in suspensions of ovine trichostrongylid larvae produced in faecal cultures artificially contaminated with the bacterium

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Presence of *Mycobacterium avium* subspecies *paratuberculosis* in suspensions of ovine trichostrongylid larvae produced in faecal cultures artificially contaminated with the bacterium

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A reference strain of *Mycobacterium avium* subspecies *paratuberculosis* was added to faecal larval cultures of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis*. Samples of the larvae produced were cultured for the presence of the bacterium in modified BACTEC 12B medium, both before and after exposure to gamma irradiation. The water used to wash the larvae off the faecal cultures was also tested for the presence of the bacterium. Positive growth was confirmed as *M avium* subspecies *paratuberculosis* by IS900 polymerase chain reaction and restriction endonuclease analysis of the product. *M avium* subspecies *paratuberculosis* was detected in the unirradiated larval suspensions and wash waters of all three nematode species, and in the irradiated *H contortus* larval suspension.

VETERINARY parasitology laboratories process faecal samples from animals that may harbour a range of intestinal pathogens. These laboratories also commonly produce infective third-stage larvae of the parasitic nematodes of sheep by faecal culture. They may be produced either as part of a disease investigation, for example of illthrift, or to produce larvae to infect other sheep for experimental purposes, either in pens or in the field. In Australia, sheep on commercial sheep farms are also deliberately infected with larvae as a part of programmes to select nematode-resistant sheep (Woolaston and Eady 1995). The worsening problem of anthelmintic resistance in ovine gastrointestinal nematodes (Overend and others 1994, Sangster 1999) has rekindled interest in the use of radiation-attenuated larval vaccines (Bain 1999, LeJambre and others 1999). These vaccines, which are administered orally, may also rely on the production of infective larvae in faecal larval cultures. Irradiated *Dictyocaulus viviparus* larvae, used in a commercial vaccine for bovine bronchitis, are also produced by coproculture techniques and administered orally (Bain 1999).

The larval culture technique involves the collection of faecal material from donor sheep which are shedding eggs of a particular nematode parasite, and the incubation of the material in a warm, humid environment. During the incubation the nematode eggs hatch, feed on bacteria in the faecal material and moult twice to third-stage larvae (Donald and others 1978). The final moult is not complete and the third-stage larva is retained within the sheath of the second-stage larva and does not feed. In the natural setting third-stage larvae migrate out of the faecal mass and on to vegetation, where they are consumed by grazing animals.

Ovine Johne's disease is a chronic debilitating disease of sheep caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis*. It results in a granulomatous enteropathy of the large and small intestine. The bacteria are shed in large numbers in the faeces of sheep clinically affected with the disease and in smaller numbers from animals with a subclinical infection (Carrigan and Seaman 1990). The disease is characterised by a long incubation period and infected animals may shed the bacterium for several months before clinical signs appear. Ovine Johne's disease is considered an economically important disease in Australia and a national control programme is under way to halt its spread; similar programmes exist in other countries. Because parasitic nematode larvae are in contact with and feed on faecal bacteria it is possible that they might become contaminated with *M avium* subspecies *paratuberculosis* and transmit the bacterium between farms, either in parasitological research trials, as the result of a larval challenge to select nematode-resistant sheep, or in vaccine trials with irradiated larvae.

The aim of this study was to determine whether *M avium* subspecies *paratuberculosis* added to larval cultures of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis* was able to contaminate the resulting larval preparations. The effect of sublethal gamma irradiation of the larvae, as used for the production of the irradiated larval vaccine, on the viability of the bacterium was also assessed.

MATERIALS AND METHODS

Faecal samples

Faeces were collected for 24 hours from sheep infected with either *H contortus*, *O circumcincta* or *T colubriformis*. The sheep were housed on wire-slatted floors and thought to be free of infection with *M avium* subspecies *paratuberculosis*, because an agar gel immunodiffusion test on serum (Stephens 1987) was negative, and radiometric culture of faecal samples (Whittington and others 1998) failed to isolate the organisms. The faecal material was stored at 4°C until used for the larval cultures. Just before the cultures were established, two faecal pellets were removed from each bulk faecal sample for culture for *M avium* subspecies *paratuberculosis*, either directly or after gamma irradiation.

Mycobacterium avium subspecies *paratuberculosis*

M avium subspecies *paratuberculosis* strain 316V was cultured in Watson Reid medium for nine weeks at 37°C and harvested by centrifugation. A bacterial suspension was prepared in sterile saline at a final concentration of approximately 10^8 cells/ml by comparison with a McFarlane standard. A 500 µl sample of the suspension was cultured for *M avium* subspecies *paratuberculosis* to ensure the viability of the organism, and another 500 µl sample was gamma irradiated.

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Bulk larval culture for nematode larvae

Larval cultures were set up in triplicate in 500 ml glass jars. Sufficient faecal material was added to each jar to one-third fill it, followed by 5 ml of the *M avium* subspecies *paratuberculosis* suspension (approximately 5×10^8 cells) and a small amount of water, and the faecal pellets were left for half an hour to soften. The pellets were then broken up thoroughly with a metal spatula and mixed with an equal volume of vermiculite. The faecal mixture was gently compressed into the bottom of each jar, the internal sides of the jars were washed with a small amount of water, the lids placed loosely on the jars and the cultures incubated at 25°C for seven days in the dark.

The cultures were then placed in light for an hour to stimulate the larvae to migrate up the sides of the jars. The larvae were harvested by washing the internal surface of each jar into a separate 500 ml glass container. The harvested larvae were allowed to settle for several hours, after which a 5 ml sample of wash water was removed from each jar for culture for *M avium* subspecies *paratuberculosis*. The larvae were then filtered through a Baermann apparatus (100 µm), the larval suspensions were divided in two, and one sample was cultured for *M avium* subspecies *paratuberculosis* immediately and the other gamma irradiated before being cultured in the same way.

Gamma irradiation

The faecal, larval and the *M avium* subspecies *paratuberculosis* samples were gamma irradiated by the Australian Nuclear Science & Technology Organisation, Lucas Heights Research Laboratories, Sydney, Australia. The radiation dose was 400 Gy. In a previous study this dose was found to be optimal for the preparation of an irradiated *H contortus* larval vaccine (J. B. Lloyd and C. Fitzgibbon, unpublished observations).

Culture for *Mycobacterium avium* subspecies *paratuberculosis*

The two faecal pellets from each bulk faecal sample, one irradiated and one not irradiated, the two 500 µl samples of *M avium* subspecies *paratuberculosis*, one irradiated and one not irradiated, the larval suspensions (nine irradiated and nine not irradiated) and the nine wash water samples were cultured for the presence of *M avium* subspecies *paratuberculosis* in modified BACTEC 12B medium (Becton Dickinson) (Whittington and others 1998, 1999). Briefly, 2 g of faeces was placed in a 15 ml polypropylene tube and mixed with 10 to 12 ml of sterile normal saline. After mixing, the tube was allowed to stand for 30 minutes at room temperature. Five ml of the surface fluid was transferred to a fresh tube containing 25 ml 0·9 per cent hexadecylpyridinium chloride (HPC) (Sigma) in half-strength brain heart infusion broth (Oxoid) and allowed to stand at 37°C for 24 hours. The suspensions and wash water were added directly into HPC and centrifuged at 900 *g* for 30 minutes. The pellet was collected and resuspended in 1 ml sterile water with vancomycin (100 µg/ml), nalidixic acid (100 µg/ml) and amphotericin B (50 µg/ml) (Sigma) and incubated for 48 to 72 hours at 37°C. One hundred microlitres of the resulting sediment was inoculated into each culture vial which contained Middlebrook 7H12 medium (BACTEC 12B; Becton Dickinson) with 200 µl PANTA PLUS (Becton Dickinson), 1 ml egg yolk, 5 µg Mycobactin J (Allied Monitor) and 0·7 ml water. The vials were incubated at 37°C for 12 weeks. The growth index was determined weekly by using an automatic ion chamber (BACTEC 460; Johnston Laboratories) and samples were collected from these cultures for polymerase chain reaction (PCR) when the growth index was greater than 200. *M avium* subspecies *paratuberculosis* was identified by IS900 PCR and restriction endonuclease analysis of the product (Whittington and others 1999).

TABLE 1: Presence (+) or absence (–) of viable *Mycobacterium avium* subspecies *paratuberculosis* in cultures containing sheep faeces and nematode larvae and samples of wash water from sheep faeces inoculated with *M avium* subspecies *paratuberculosis*

Sample	Before irradiation	After irradiation
M avium subspecies *paratuberculosis* suspension	+	+
Faeces containing *H contortus* eggs	–	–
Faeces containing *T colubriformis* eggs	–	–
Faeces containing *O circumcincta* eggs	–	–
H contortus larvae (3 replicates)	+,+,+	+,–,–
T colubriformis larvae (3 replicates)	+,+,+	–,–,–
O circumcincta larvae (3 replicates)	+,+,–	–,–,–
H contortus wash water (3 replicates)	+,–,–	Not done
T colubriformis wash water (3 replicates)	+,–,–	Not done
O circumcincta wash water (3 replicates)	+,+,–	Not done

RESULTS

M avium subspecies *paratuberculosis* was cultured from all three of the replicates of unirradiated *H contortus* and *T colubriformis* larvae, from two of the three replicates of unirradiated *O circumcincta* larvae and from one of the three replicates of irradiated *H contortus* larvae (Table 1). It was also cultured from two of the three replicates of *O circumcincta* wash water and from one of the three replicates of the *H contortus* and *T colubriformis* wash waters.

The time to develop a growth index is correlated with the number of viable *M avium* subspecies *paratuberculosis* present. All of the unirradiated larval suspensions produced a growth index of 999 (the highest possible reading) in the BACTEC system after seven weeks of culture (Table 2). At this time the *M avium* subspecies *paratuberculosis* positive control samples, both irradiated and unirradiated, also had growth indices of 999. In contrast only one of the four wash water samples which produced growth had reached the maximum growth index at this time. The sample of irradiated *H contortus* larvae also did not reach the maximum growth index until several weeks later. During the 12 weeks of incubation of the *M avium* subspecies *paratuberculosis* cultures none of the negative control sample faecal pellets from the nematode-infected sheep produced a positive growth index.

DISCUSSION

This is the first report of the potential for parasitic nematode larvae of sheep to become contaminated with *M avium* subspecies *paratuberculosis*. The results have practical significance for parasitology laboratories. Faecal samples are often received at diagnostic laboratories as part of an investigation of illthrift in sheep, for which both ovine Johne's disease and intestinal parasitism may be differential diagnoses. Care must be taken in the processing of these samples, especially if the laboratory also produces nematode larvae for use in research trials, particularly field trials. At the Elizabeth Macarthur Agricultural Institute a programme to prevent cross-contamination has been put in place. The two functions of the parasitology laboratory, diagnostic parasitology and research, have been physically separated with no exchange of laboratory equipment or glassware between the two. Separate facilities for storing specimens, processing faecal samples, culturing larvae, and washing glassware have been established. In addition, consideration has been given to the treatment of the effluent from the diagnostic laboratory.

Research parasitology laboratories that do not receive diagnostic specimens must still ensure that the donor sheep

TABLE 2: Time after establishment of cultures for *M avium* subspecies *paratuberculosis* when samples first produced a growth index of 999

Sample	Week of culture 4	5	6	7	8	9	10	>10
M avium subspecies *paratuberculosis* suspension				1,R				
H contortus larvae	1,2,3							R1
T colubriformis larvae	1	3		2				
O circumcincta larvae				1,2				
H contortus wash water								2
T colubriformis wash water	3							
O circumcincta wash water							1	2

1 Replicate 1, 2 Replicate 2, 3 Replicate 3, R Irradiated

used to produce larvae are free of *M avium* subspecies *paratuberculosis* infection. This is of particular importance if the larvae are used to infect sheep in the field. Measures to ensure freedom from *M avium* subspecies *paratuberculosis* infection could include obtaining animals only from flocks which are known to be free of the infection, housing animals on wire-slatted floors from birth, using animals less than 18 to 24 months of age and only infecting donor sheep with a larval inoculum that has been shown to be free of *M avium* subspecies *paratuberculosis*.

The results indicate that 400 Gy of gamma irradiation was not sufficient to inactivate *M avium* subspecies *paratuberculosis* in suspension or in contaminated larvae. However, it is not known whether sufficient bacteria survive the irradiation to infect a sheep when they are administered with nematode larvae. However, this result suggests that care will be needed during the development of irradiated larval vaccines to ensure that contamination with *M avium* subspecies *paratuberculosis* does not occur. Measures to prevent contamination, as described above, and the testing of batches of the irradiated larvae before their release to ensure that they are free from contamination may both be required.

The experiments do not indicate where in the larval suspensions the *M avium* subspecies *paratuberculosis* bacteria are located. Possible sites include within the nematode larvae themselves, either within the digestive tract or larval tissues, between the larva and its external sheath, adherent to the outside of the sheath or free in suspension with the larvae. The filter used in the Baermann apparatus (100 µm) would not have been small enough to remove free bacteria from the larval suspensions. More of the larval suspensions than in the wash waters were positive for *M avium* subspecies *paratuberculosis* growth, which suggests that the bacteria were associated with the larvae. The generally shorter time required to establish positive growth in the larval suspensions than in the wash waters also suggests that more bacteria were associated with the larvae than were free in suspension. In liquid culture mycobacteria tend to form clumps which could have settled with the larvae. It is therefore possible that the bacteria were more associated with the larvae than the wash water because of the several hours the larval suspensions were left to settle. From the point of view of the risk of spreading *M avium* subspecies *paratuberculosis* the location of the bacteria is irrelevant, because both the larvae and the suspension liquid are administered to sheep in parasitology trials.

The results also suggest that parasitic nematode larvae may play a role in the transmission of *M avium* subspecies *paratuberculosis*. Nematode larvae have been implicated in the transmission of *Salmonella typhimurium* in mice (Bottjer and others 1978) and of atypical mycobacteria in cattle (Mota and others 1987). First- and second-stage larvae of the parasitic nematodes of sheep live within the faecal mass, feeding on faecal bacteria. Third-stage larvae migrate in a water film from the faecal mass on to vegetation, from where they are consumed by grazing sheep. This could be a mechanism for the transmission of *M avium* subspecies *paratuberculosis* from within the faecal mass to the surrounding pasture, and from there to grazing sheep. This may happen regardless of whether the bacteria are associated with the larvae or free in the water film, because the sinusoidal action of the larvae as they migrate is likely to enhance the movement of any free bacteria. However, the transmission is likely to be more effective if the bacteria are closely associated with the larvae. Once ingested the larvae may contribute to the bacterial infection of their host in several ways. The shedding of the larval sheath may release the bacteria that then become available in the gut lumen. Once the sheath has been shed third-stage larvae either enter or become intimately associated with the gastrointestinal mucosa, where they moult to fourth-stage larvae (Donald and others 1978), possibly allowing the bacteria to enter the mucosa.

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