Routes of Intraspecies Transmission of *Mycobacterium avium* subsp. *paratuberculosis* in Rabbits (*Oryctolagus cuniculus*): a Field Study

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Rabbits have been increasingly linked to the persistence of paratuberculosis (Johne’s disease) in domestic ruminants in the United Kingdom. The aims of this study were to determine the routes of intraspecies transmission of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in rabbits and to estimate the probability of transmission via each route, in order to gain understanding of the dynamics of MAP in this host.

Rabbits were sampled from two sites where MAP had previously been isolated from the livestock and rabbit populations. No pathology was noted in any animals, but the overall prevalence of MAP in rabbits was high at both sites studied, 39.7% and 23.0%, respectively. MAP was isolated from the testes, uterus, placenta, fetuses, and milk. This is the first time that the bacterium has been isolated from any of these tissues in a nonruminant wildlife species. These results suggest that transmission may occur vertically, pseudovertically, and horizontally.

**Paratuberculosis, also known as Johne’s disease, is a chronic, usually fatal enteritis of all ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease causes significant economic losses to the agricultural industry worldwide (5, 27), and there are associated welfare issues for infected animals. It is notoriously difficult to control in domestic ruminant populations. Animals are generally thought to become infected within the first few months of life (27). However, clinical signs are usually seen only in adults due to the long incubation period of the disease (6).

Historically it was thought that the infection was found only in ruminants. However, recent studies have shown that it is present in a number of nonruminant wildlife species, including the fox (*Vulpes vulpes*), stoat (*Mustela erminea*), crow (*Corvus corone*), Norway rat (*Rattus norvegicus*), and rabbit (*Oryctolagus cuniculus*) (2, 3, 13, 14). Of these species, the rabbit is thought to pose the greatest risk of inter species transmission of MAP. This is because of the combination of the high prevalence of MAP infection in rabbits (up to 63%) (3, 13, 14) and the high rate of excretion of bacteria in their feces (up to 106 CFU per gram of feces, which could constitute an infective dose for a ruminant) (8), along with the lack of behavioral avoidance of rabbit fecal pellets by ruminants while grazing (8, 17). All of these factors lead to a high potential for interspecies transmission of MAP from rabbits to grazing ruminants. Furthermore, MAP isolated from rabbits is morphologically and genetically indistinguishable from that found in ruminants (13), and calves experimentally inoculated with the rabbit strain of the organism have subsequently become infected with paratuberculosis (4).

The presence of a nonruminant wildlife reservoir for MAP could explain why the disease has proved difficult to control in livestock (9). Determining the dynamics of the disease in rabbits is central to determining if rabbits are a true reservoir for the disease, i.e., whether the disease can persist in the rabbit population without input from any other species. The aims of this study were to determine the routes of transmission of MAP in a natural population of rabbits and to estimate the probability of transmission via each of these routes in order to understand the dynamics of MAP in rabbits.

It is intuitive to assume that transmission of MAP in rabbits may occur via the same routes as in ruminants, where there are three main routes through which intraspecies transmission of MAP may occur. These are vertically, pseudovertically, and horizontally. Vertical transmission may occur with calves becoming infected in utero (24, 26); transplacental transmission occurs in up to 40% of clinically infected and 8.6% of subclinically infected dams (27). Pseudovertical transmission is thought to occur through suckling. The organism is shed in the colostrum and milk of infected dams (10, 25, 26, 28), and transmission may also occur through calves suckling on fecally contaminated udders. Horizontal transmission may occur in two ways: the infection could potentially be transmitted sexually, as the bacterium has been isolated from the semen of bulls (1), or via the fecal-oral route, which is considered to be the main mode of transmission in ruminants (27). Therefore, these
routes were used as a focus for the potential routes of transmission in populations of wild rabbits.

MATERIALS AND METHODS

Collection of samples. Four hundred and eighty-seven rabbits in total were sampled from the population following a standardized random-sampling regime between April 2002 and May 2004 from two sites in Perthshire, Scotland. This was achieved by applying the same monthly sampling effort during systematic searches of the study sites. MAP has previously been isolated from livestock and rabbits at both sites (13, 14). Random sampling (across age, sex, and reproductive classes of animals) of the rabbit population was carried out at site 1 to provide (i) a baseline background prevalence and (ii) tissues and excreta for testing for the presence of MAP. This sample did not include sufficient numbers of young rabbits (<3 months old) or pregnant females, both of which are necessary to estimate the rates of vertical and pseudovertical transmission; therefore, a second study site was added. Random sampling of the rabbit population was carried out at site 2 (i) to provide a baseline background prevalence for this site and (ii) to increase the sample size of young rabbits and pregnant females.

Despite the inclusion of the second site, the sample sizes of young rabbits and pregnant females remained small; therefore, further (targeted) sampling of these two age classes was carried out at both sites to minimize the variation of the proportion of transmission via vertical and pseudovertical routes.

Gross postmortem procedures. Postmortems were carried out within 18 h of sampling taking place. Rabbits were tagged with an individual identification number and weighed. New sterile disposable scalpels, forceps, pipettes, and needles were used for each rabbit, and the work area was disinfected after each rabbit to reduce the risk of cross-contamination. The postmortems followed the procedure below in order to reduce the risk of cross-contamination from the gut to any other sample taken. (i) Eyeballs were removed and placed in 10% formal saline for age determination purposes (see below). (ii) The skin was removed from the ventral abdomen; if the rabbit was a lactating female, milk was collected using a fine pipette. (iii) An incision was made in the abdominal wall, and the reproductive organs, i.e., the testes, uterus, and, in gestating females, the fetuses were removed. (iv) Hard feces, if present, were recovered from the colon. (v) The mesenteric lymph node was removed and put together with a section from the ileum, corpus caeci, appendix, and a third of the sacculus to provide the pooled gut tissue sample. (vi) A second third of the sacculus and a second section from the ileum, corpus caeci, appendix, and a third of the sacculus to provide the second section of the appendix were used to prepare mucosal smears for Ziehl-Neelsen staining. (vii) The final third of the sacculus and a third section of the appendix were placed in 10% formal saline for histopathological examination.

Histopathology. Sections of the sacculus and appendix from a subset of the sample were taken for histopathological evaluation. The tissues were fixed in 10% formal saline for a minimum of 24 h, trimmed, dehydrated through graded alcohol, embedded in paraffin wax, and sectioned (5 µm). The sections were stained with hematoxylin and eosin for routine histopathological examination and to test for acid-fast bacilli by the Ziehl-Neelsen method. Culture. Tissue pool, fecal, milk, fetal, testes, uterus, and placental (pool of cotyledons) homogenates were prepared following the protocol of Greig et al. (14). Fetuses were removed from the placenta and weighed; those weighing less than 3 g were processed as a pool of all fetuses from that litter, and those above 3 g were eviscerated, and the liver, gut, and stomach were processed as a pool for each fetus. Briefly, for all homogenates, 1 g of feces or 0.5 cm³ of finely chopped tissues was homogenized in 5 ml of sterile distilled water with a Colworth Stomacher 80 (Seward Medical, London, United Kingdom). The homogenates were decontaminated by adding 5 ml of 1.5% cetyl pyridinium chloride and allowed to stand overnight at room temperature to allow particulate material to settle. The supernatants were centrifuged at 4,000 rpm for 30 min, and each pellet was resuspended in 5 ml of sterile distilled water. The centrifugation step was repeated, and each pellet was resuspended in 500 µl of sterile distilled water. Two slopes of Middlebrook 7H11 agar supplemented with Selectatabs (amphotericin B, polymixin B, carbenicillin, and trimethoprim; code MS24; MAST Laboratories Ltd., Merseyside, United Kingdom), 10% Middlebrook oleic acid- albumin-dextrose-catalase enrichment medium (Difco, Surrey, United Kingdom), and 2 µl of mycobactin J (Allied Monitor, Fayette, Mo.) per ml were inoculated with 110 µl of the prepared suspension. The cultures were incubated for up to 16 weeks at 37°C and examined regularly for bacterial growth.

PCR. Mycobacterium avium subsp. paratuberculosis from the cultures was confirmed using the IS900 insertion sequence. Briefly, 100 µl of sterile distilled water was inoculated with a single colony of a positive culture. DNA was extracted from the bacterial suspension by heating it at 100°C for 10 min and then centrifuged at 13,000 rpm for 2 min; 5 µl of the supernatant bacterial suspension was analyzed by PCR using electrophoresis in a 2% agarose gel in 1× Tris-borate-EDTA buffer. The gels were stained in ethidium bromide (5 µg/ml) and visualized by UV light transillumination.

Age determination of rabbits. The eye lenses were used to determine the ages of rabbits sampled by following the protocol described by Wheeler and King (32). Briefly, the eyeballs were stored in 10% formal saline for a minimum of 14 days before the lenses were removed and oven dried at 85°C for 7 days. The mean dry weight of the lenses was then used to calculate the age of the rabbit using the equation given by Wheeler and King (32).

Statistical analysis. Generalized linear models using a negative binomial distribution with a logit link function were used to determine differences in prevalence between age groups (samples were divided into five age groups: <3 months, juveniles; >3 and ≤6 months, potentially sexually mature but not likely to be breeding; >6 and ≤12 months, likely to be first-time breeders; >12 and ≤24 months, performing the majority of breeding; and >24 months, as age determination is not accurate above 24 months) and sexes (divided into three groups: male, nonpregnant female, and pregnant female).

Probabilities/rates of transmission. A maximum likelihood fitting procedure was used to derive probabilities of vertical/pseudovetical and horizontal transmission from the field data at study site 1. In order to model the variation of the mean prevalence with age, it was assumed that both the number of individuals at any given age and the number of infected individuals at any given age remain constant, at least on the time scale of an individual’s lifetime. This is consistent with the finding that the overall infection prevalence in rabbits did not increase across the years of sampling (18). Given this assumption, it was then possible to pool the prevalence data taken on each visit and treat the inferred prevalence for each age as being equal to the prevalence that would be measured if it was possible to track a cohort of individuals from birth to death, measuring the prevalence in that cohort. A model for the spread of disease over time in a group of individuals exposed to a constant level of infection can therefore be used.

The model was constructed by assuming a two-stage infection process; individuals are exposed only to vertical and pseudovertical infection until time $t_0$, when all vertical/pseudovertical infection ceases and they become exposed to horizontal infection by infected rabbits they are in contact with. The absence of sufficient data from preweaned individuals prevented using a detailed model of the horizontal processes, so the combined effects of vertical and pseudovertical transmission were represented by a single probability, $P_v$, that individuals are infected at $t_0$. The horizontal infection process was modeled as a homogeneous Poisson process (representing the simplest mathematical form for horizontal infection within a homogeneously mixing social group of rabbits; see below for the group size) with a constant infection rate in which $I$ is the (constant) number of infected individuals in the population as a whole and $\lambda_v$ is the per capita rate of infection.

$$\lambda_v = \beta I$$

(1)

In a homogeneous Poisson process, the probability that an event occurs in the time interval $(0, t)$ is

$$1 - e^{-\lambda t}$$

(2)

Including the effect of vertical transmission, there are two ways that an individual could be infected at time $t$: by being infected vertically/pseudovertically from its mother, with probability $P_v$, or horizontally, with probability

$$(1 - P_v)(1 - e^{-\lambda t})$$

(3)

while in order to escape infection up to time $t$, an individual must avoid infection through both routes, leading to a probability

$$1 - P_v e^{-\lambda t}$$

(4)

Combining these probabilities with the data, where $t_i$ is the age of the $i$th rabbit and $y_i$ is a binary value indicating whether that individual was infected, the likelihood

$$L(\lambda_v, P_v, \beta, d) = \prod_{i=1}^{y_i} \left[ P_v + (1 - P_v)(1 - e^{-\lambda v t_i}) \right] + \prod_{i=1}^{1 - y_i} \left[ (1 - P_v) e^{-\lambda v t_i} \right]$$

(5)

is formed, which was maximized in order to infer values for the parameters $\lambda_v$ (per capita rate of infection) and $P_v$ (vertical/pseudovertical transmission rate).
RESULTS

Of the initial random samples of all rabbit categories, MAP was isolated from the gut pool samples of 100 (39.7%) of 252 from site 1 and 29 (23.0%) of 126 from site 2. None of the animals displayed any visible signs or lesions indicative of paratuberculosis at postmortem examination.

The results of culture and PCR for the reproductive organs, feces, and milk from the 100 gut pool-positive rabbits at site 1 are given in Table 1. MAP was isolated from 21.5% (11/52) of testes, 17% (8/47) of uteri, 100% (8/8) of feces, and 14.8% (4/27) of milk samples cultured from gut pool-positive animals. The culture and PCR results for all pregnant females’ gut pool samples, placentas, and litters of fetuses from both sites are shown in Table 2. As in some cases the fetuses were too small to be processed individually and were therefore processed as a pool, the results are shown for the litter, not individual fetuses within the litter. Of the pregnant females sampled, 36.8% (14/38) from site 1 and 53.4% (31/58) from site 2 were gut pool culture and PCR positive. Of these individuals, 7.1% (1/14) of litters from site 1 were culture, but not PCR, positive for Mycobacterium spp. and 3.2% (1/31) of litters from site 2 were culture and PCR positive. However, the mother of this litter had perforated intestines, and therefore, cross-contamination at collection cannot be ruled out.

The prevalence of MAP varied between age groups at both site 1 (deviance ratio [dr], 4.76; df, 4; P < 0.001) and site 2 (dr, 0.12; df, 1; P < 0.001). Age group 1 (≤3 months) had a significantly lower prevalence than any other age group at both sites (11.1% [3/27] at site 1 and 3.2% [2/63] at site 2) (Fig. 1).

The maximum likelihood procedure (Fig. 2) generated values for λ₀ of 0.037 and for Pᵥ of 0.14 when a weaning age (tᵥ) of 1 month was used. These values can be expressed in terms of the underlying transmission probabilities.

This per capita rate of horizontal infection per month (λ₀) is specific to our study site and will vary depending on the number of infectious (I) and susceptible animals in regular contact. The generic horizontal transmission coefficient per month (β) can be estimated as follows:

\[ \lambda_0 = \beta I = \beta(NP) \]
\[ \beta = \lambda_0/(NP) \]  
(6)

where P is the overall prevalence.

At the time the samples were collected, the population density at study site 1 was very low, as evidenced by the large proportion of disused warrens (41.2% disused [J. Judge, unpublished data]). At low population densities, where nest sites are not limited, females do not tend to form groups with other females (7, 23). This suggests that the majority of groups at study site 1 constituted only a single adult pair, in which case β could be equal to or greater than λ₀. During the breeding season, these groups would be larger due to the inclusion of the adult pair’s offspring; however, infants would not be expected to be involved in horizontal transmission because they do not spend much time out of the nest until they are weaned. One adult female produces an average of 20 kits per year (29, 30, 36), 50% of which will not reach weaning age, and only 53% of those that reach weaning will survive to 80 days old (22). Therefore, a realistic group size during the breeding season (excluding infants) would be seven individuals. Conservatively, the β value would therefore be expected to be within the range of 0.013 to 0.046.

The proportion of individuals entering the population after being weaned (at 1 month old) that were infected via vertical and/or pseudovertical transmission (Pᵥ), estimated from the maximum likelihood procedure, was 0.14. As only offspring from infected does can be infected vertically or pseudovertically, the probability of transmission via these routes can be calculated from the proportion of infected juveniles entering the population after being weaned and the proportion of infected females of reproductive age. There was no significant difference in the prevalence of MAP between sexes at either site (dr, 0.12; df, 1; P > 0.1 for site 1 and dr, 1.63; df, 1; P > 0.1 for site 2); therefore, it can be assumed that equal percentages of males and females were infected with MAP. At site 1, 42.9% (85/198) of adults of reproductive age (i.e., >6 months old) were MAP positive. Assuming that there is no effect of MAP infection on either reproductive output or juvenile survival, this gives a probability of infection via vertical and/or pseudovertical transmission of up to 0.326 (14% of young females (7, 23). This suggests that the majority of groups at study site 1 constituted only a single adult pair, in which case β could be equal to or greater than λ₀. During the breeding season, these groups would be larger due to the inclusion of the adult pair’s offspring; however, infants would not be expected to be involved in horizontal transmission because they do not spend much time out of the nest until they are weaned. One adult female produces an average of 20 kits per year (29, 30, 36), 50% of which will not reach weaning age, and only 53% of those that reach weaning will survive to 80 days old (22). Therefore, a realistic group size during the breeding season (excluding infants) would be seven individuals. Conservatively, the β value would therefore be expected to be within the range of 0.013 to 0.046.

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TABLE 1. Culture and PCR results from the reproductive organs, feces, and milk from the 100 gut pool-positive rabbits at site 1

<table>
<thead>
<tr>
<th>Source</th>
<th>No. sampled</th>
<th>No. culture positive</th>
<th>No. PCR positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes</td>
<td>52</td>
<td>11</td>
<td>11</td>
<td>21.5</td>
</tr>
<tr>
<td>Uterus</td>
<td>47</td>
<td>8</td>
<td>8</td>
<td>17.0</td>
</tr>
<tr>
<td>Feces</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>100.0</td>
</tr>
<tr>
<td>Milk</td>
<td>27</td>
<td>4</td>
<td>4</td>
<td>14.8</td>
</tr>
</tbody>
</table>

TABLE 2. Culture and PCR results for the gut pool samples, placentas, and litters of fetuses taken from pregnant females from both sites

<table>
<thead>
<tr>
<th>Source</th>
<th>Site 1</th>
<th></th>
<th></th>
<th>Site 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. sampled</td>
<td>No. culture positive</td>
<td>No. PCR positive</td>
<td>%</td>
<td>No. sampled</td>
<td>No. culture positive</td>
</tr>
<tr>
<td>Pregnant females</td>
<td>38</td>
<td>14</td>
<td>14</td>
<td>36.8</td>
<td>58</td>
<td>31</td>
</tr>
<tr>
<td>Placenta</td>
<td>14</td>
<td>3</td>
<td>3</td>
<td>21.4</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Litters</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>71</td>
<td>31</td>
<td>1</td>
</tr>
</tbody>
</table>

a Results for placentas, and litters are given only for females that were gut pool positive.
b All three fetuses from this litter were individually processed, and all three were both culture and PCR positive. However, the mother had perforated intestines, and therefore cross-contamination at collection cannot be ruled out.
infected when entering the population at 1 month/42.9% of infected females of reproductive age).

DISCUSSION

The main aims of this study were to determine the routes of transmission of MAP in rabbits and to estimate the probability of transmission via each route. Overall, the prevalence of MAP in rabbits was high at both sites studied. MAP was isolated from the testes, uterus, fetuses, placenta, and milk, which is the first time it has been isolated from these samples in any non-ruminant wildlife species. The presence of MAP in the placenta and fetuses suggests that vertical transmission of MAP may occur in rabbits. The isolation of MAP from both milk and feces suggests that there is a potential for pseudovertical transmission to occur, and its presence in testes, uterus, and feces suggests the potential for horizontal transmission.

As stated earlier, transmission of MAP occurs vertically in utero in cattle. Cattle have a syndesmochorial placenta, in which the lining epithelium of the uterus is the only maternal tissue that is eroded (31); therefore, there is little contact between the maternal blood and the fetus. In contrast, rabbits have a hemoendothelial placenta, where the endothelium of the capillaries of the chorion come into direct contact with the maternal blood (31). This suggests that there would be much greater potential for vertical transmission of MAP in rabbits than in cattle. However, in this study, only 7.1% (1/14) at site 1 and 3.2% (1/31) at site 2 of litters from MAP-positive does were culture positive for *Mycobacterium*. Furthermore, the results from the fetal tissues were not conclusive, as the culture- and PCR-positive fetuses from site 2 came from a mother whose intestines had been perforated, and therefore, cross-contamination cannot be ruled out. Also, the culture-positive fetus from site 1 was not PCR positive, and therefore, we cannot be certain that it was MAP and not another mycobacterium. Further research is needed to quantify the rate of vertical transmission of MAP in rabbits.

Pseudovertical transmission in rabbits may occur through the ingestion of contaminated milk while suckling and/or the ingestion of contaminated fecal pellets during the weaning process, as does deposit fecal pellets in the nest in the days

FIG. 1. Prevalence of infection with MAP by age class at sites 1 (shaded bars) and 2 (stippled bars).

FIG. 2. Maximum likelihood function fitted to the data. The data are categorized in age ranges of 2 months.
prior to weaning for the kits to ingest (15). Using the prevalence of MAP in rabbits entering the population at weaning (approximately 1 month old) and taking into account the prevalence of MAP in females of breeding age as an indication of the rate of vertical and/or pseudovertical transmission, the probability of infection via these routes is up to 0.326 for offspring of infected females.

It is highly likely that in the majority of cases the level of MAP infection in fetuses and young rabbits is very low, due to the slow-growing nature of the bacteria. The ability to detect these low levels of infection may be confounded by the under-detection of the bacteria due to the low sensitivity of the culture. The sensitivity of the solid-medium culture method used here has been determined to be approximately 30% (11, 19, 33) for individuals with low levels of infection, as would be expected in fetuses and juveniles. Therefore, the actual prevalence of MAP could be more than three times that detected here. If the culturing method detected bacteria in only 30% of samples in which they were present, the true prevalence of MAP in juveniles at site 1 may actually have been 46%, above the mean prevalence for that site. Consequently, it may be that the methods of detection of MAP at low levels were not adequate and that vertical and pseudovertical transmission rates are much higher than suggested from the culture results obtained. Detection at low levels of infection may be further confounded by the effects of the decontamination process used during culturing, which may kill up to 2 log_{10} bacteria (16, 20, 21, 34). If this were the case, transmission of MAP from infected does to their offspring via vertical and pseudovertical routes would be much greater.

Unless vertical/pseudovertical transmission of a pathogen is a certainty, these routes of transmission alone cannot maintain infection in a host population. Horizontal transmission is necessary for the infection to persist without interspecies transmission and for rabbits to constitute a true reservoir for MAP. Once animals have been weaned, they would be susceptible to infection only via horizontal routes or through interspecies transmission. There is a rise in the level of infection in rabbits over 1 month old, equating to a probability of an individual becoming infected via horizontal transmission (which would also include interspecies transmission) of up to 0.037 per month. This level of horizontal transmission suggests that there is a high probability of MAP infection over the average 18-month life span of a wild rabbit.

The field data suggest two potential routes of horizontal rabbit-to-rabbit transmission of MAP, the fecal-oral route and sexual transmission. It has already been determined that infected rabbits shed a high number of the bacteria in their feces (up to 10^6 CFU/g feces) (8); therefore, there is a potential for horizontal transmission via this route. Interspecies transmission is only likely to occur via the fecal-oral route. However, there do not appear to be any records of adult rabbits deliberately ingesting the feces of other individuals. Furthermore, rabbits are highly selective grazers (12) and are unlikely to ingest fecal material accidentally while grazing. This is in complete contrast to the risks of rabbit-to-cattle transmission, as grazing cattle show no avoidance of rabbit feces and are exposed to potentially infective doses of MAP from rabbits via the fecal-oral route on a daily basis (17). Consequently, the risk of transmission, whether inter- or intraspecies, to rabbits via the fecal-oral route would seem low compared to the risk to cattle from rabbits. However, it is possible that the bacteria may remain viable in the environment for up to a year (5, 35), long after the fecal pellet will have weathered away, and therefore, there is a potential for rabbits to ingest contaminated vegetation.

The recovery of MAP from both the uterus and testes of rabbits suggests that there is a theoretical potential for sexual transmission to occur. Further investigation of this route of transmission in rabbits needs to be undertaken. Similarly, the isolation of MAP from the semen of bulls suggests that sexual transmission in cattle should not be ruled out (1). If sexual transmission is confirmed in rabbits, it may be a key route of MAP transmission, as it would help maintain infection in rabbit populations and thus increase the likelihood of rabbits contributing a reservoir of MAP infection.

The results provide evidence of the potential for transmission of MAP in rabbit populations via vertical, pseudovertical, and horizontal routes. The presence of these routes lends further support to the suggestion that rabbits are a significant source of MAP for livestock, as these routes of transmission help maintain MAP infection in rabbit populations and, therefore, in the environment. If rabbits are a true reservoir for MAP, it is essential that they be included in any management/control strategies for the disease in farmed ruminants.

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