

The potential role of wild rabbits *Oryctolagus cuniculus* in the epidemiology of paratuberculosis in domestic ruminants

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SUMMARY

Mycobacterium avium subspecies *paratuberculosis*, the organism responsible for paratuberculosis in cattle and sheep has been found in wild rabbits (*Oryctolagus cuniculus*) in the east of Scotland. Few studies have investigated either the level of faecal contamination by rabbits on farms, or the potential infectivity of rabbit excreta. The rate of rabbit faecal contamination deposited and the numbers encountered were estimated for 21 fields on 4 farms with a paratuberculosis problem. 7357 ± 2571 s.e.m. rabbit faecal pellets were deposited per hectare per day and up to 81 000 pellets/ha ('standing crop') were encountered in October/November 1998. Where access to rabbits was restricted, the standing crop of faeces encountered fell to 22 000 pellets/ha.

The prevalence of infection with *M. a. paratuberculosis* was assessed for 83 rabbits from the four farms. *M. a. paratuberculosis* was isolated from rabbits on all farms with an overall prevalence of 17%. Out of 17 rabbits from which urine was available, *M. a. paratuberculosis* was isolated from two – the first reported isolation from urine in wild rabbits. The mean number of colony-forming units per gram of infected rabbit faeces was $7.6 \times 10^5 \pm 5.2 \times 10^5$.

A relative estimate of the input of *M. a. paratuberculosis* onto pasture, at the stocking levels found on the four farms, showed that sheep and cattle potentially contributed 4 and 125 times more organisms/ha per day respectively than rabbits. However, rabbits could still contribute millions of *M. a. paratuberculosis* organisms per ha per day. Existing rabbit control measures on farms may be inadequate in reducing the risk of transmission to livestock.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*M. a. paratuberculosis*), the organism which causes paratuberculosis (or Johne's disease) in cattle and sheep, has been found in wild rabbits (*Oryctolagus cuniculus*) in Scotland [1]. Evidence of a relationship between

farms with a previous or current paratuberculosis problem in livestock and the presence of paratuberculosis in rabbits, has also been demonstrated [2]. Transmission of paratuberculosis is assumed to occur mainly through the faecal–oral route [3] and consequently there exists the potential for infected rabbit excreta to transmit the disease to grazing livestock. Few studies have investigated either the level of faecal contamination by rabbits on farms with a paratuberculosis problem, or the potential infectivity of rabbit excreta.

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Based on trapping and radio tracking, a density of between 10.1 and 59 rabbits/ha has been estimated for the east of Scotland [4]. Given an individual rabbit can produce between 276 and 448 faecal pellets per day [5, 6], then there is the potential for between 2787 and 26432 faecal pellets per ha per day to be deposited on grazing swards and latrines (accumulations of faeces used for social communication [7]).

As well as the numbers of rabbit faeces encountered on grazing swards, their relative infectivity compared to livestock faeces is also important when quantifying the potential disease risk they pose. Clinically affected cattle may shed in excess of 10^8 bacilli/g of faeces [8] and sheep have been reported to excrete 1.09×10^8 viable bacteria/g of faeces [9].

The 'natural' infective dose required to produce clinical disease in livestock is unknown. Consequently, estimates can be based only on the doses found to produce disease in experimentally infected animals. The minimum experimental dose required to infect calves has not been determined [10] but lambs can develop infection with doses of between 10^3 and 10^9 organisms [11]. Consequently, calves and lambs could potentially receive an infective dose from ingesting a few grams of infected livestock faeces. Since *M. a. paratuberculosis* can survive in the environment for up to a year [12], such an infective dose could be received by ingestion of grass contaminated with faeces.

The exact role of rabbits in the epidemiology of paratuberculosis is unknown. However, regardless of their role in the transmission of paratuberculosis, rabbits are considered to have a significant economic impact on agriculture [13, 14]. On pasture, experiments have demonstrated that rabbit grazing can reduce livestock weight gain and consequently production [15]. As a result many farms practice some form of rabbit control. However, the impact of such control on the numbers of faecal pellets entering grazing pasture and consequently the potential for paratuberculosis transmission is, to date, unquantified.

Below, we describe the quantity of rabbit faeces encountered on grazing pasture on four farms in the east of Scotland where *M. a. paratuberculosis* had previously been isolated from both cattle and rabbits [1, 2]. An estimate of the prevalence of infection in rabbits and their excreta is also reported. We then discuss the relative risk posed by rabbits in the transmission of paratuberculosis to livestock and the efficacy of existing rabbit control measures.

METHODS

Study farms

The four farms selected for study were at least 20 km apart: farms A and B in Perth and Kinross and C and D in Angus. All farms were mixed enterprises, with beef suckler herds and sheep flocks as well as arable land producing mainly feed barley and potatoes. Farms ranged in size from approximately 200–2000 ha.

All farms conducted some form of rabbit control, with three (farms A, B, C) attempting to maintain specific fields as 'rabbit free' by the use of rabbit netting. However, 'rabbit-free' fields were either not netted on all sides, had box traps which when not set allowed access to the fields or had been undermined by burrowing. Consequently rabbits were potentially able to enter these fields which are therefore classed as 'restricted access' (as opposed to rabbit free).

Estimating rates of pellet deposition

Twenty-one fields (six each from farms A, B and C and three from the smallest farm D which had no restricted access fields) were selected as being representative of the pasture used by grazing livestock throughout the year. Ten permanent plots (0.5×0.5 m) were established in two parallel lines, five at the 'edge' (defined as <2 m from the field fence) and approximately 10 m apart and five in the 'middle' (defined as >10 m from field fence) of each field. Plots were stratified between the edge and middle of fields to test whether pellets were deposited differently in relation to field position.

Each plot was marked by a wooden peg ($2.5 \times 2.5 \times 16$ cm) at one corner. Pegs were buried until only 0.5 cm remained above the field surface – sufficient to enable relocation while minimizing potential effects on rabbit behaviour. Each plot was cleared of all pellets present and revisited at intervals of 14 days between November 1998 and February 1999 and the number of pellets recorded. This time period was chosen because cattle had been removed from pasture to overwinter indoors and rabbit numbers were assumed to be at their most stable then, as rabbits are least likely to breed over these months [16]. Sheep were present on all 21 fields during the study period.

Estimating the 'standing crop' of rabbit faeces

The number of faeces present on grazing pasture at any given time (i.e. the 'standing crop') is the total of accumulated faeces deposited minus losses due to

Table 1. Mean numbers of rabbit faecal pellets deposited fortnightly per m² on farms in the east of Scotland (standard error means in parentheses)

Farm	8 Dec. 98	22 Dec. 98	6 Jan. 99	20 Jan. 99	3 Feb. 99	17 Feb. 99	Overall mean
A	7.5 (0.9)	5.3 (0.9)	4.5 (0.4)	6.7 (0.8)	9.1 (1.0)	6.7 (0.8)	6.6
B	14.3 (1.5)	18.6 (2.6)	17.7 (2.4)	18.3 (2.1)	16.3 (2.0)	15.0 (1.8)	16.7
C	20.7 (3.4)	18.5 (2.5)	11.8 (1.5)	15.7 (2.2)	17.1 (1.7)	12.1 (1.4)	16.0
D	1.7 (0.3)	3.5 (0.4)	2.0 (0.3)	1.3 (0.2)	1.5 (0.2)	1.9 (0.1)	2.0
Overall mean	11.1	11.5	9.0	10.5	11.0	8.9	10.3

Table 2. Mean numbers of rabbit pellets, latrines and sward heights encountered in fields in October/November 1998. (Backtransformed means are shown with 95% confidence intervals, means for sward height with standard error means.)

Farm (no. fields)	Restricted access?	Field position	Pellets/m ²	Latrines/m ²	Sward height (mm)
A					
(3)	No	Edge	3.8 (3.5–4.2)	0.05 (0.05–0.05)	48.3 (3.0)
		Middle	6.2 (5.6–6.8)	0.12 (0.12–0.12)	48.8 (1.5)
(3)	Yes	Edge	0.9 (0.7–1.0)	0.03 (0.02–0.03)	63.0 (1.9)
		Middle	2.8 (2.5–3.1)	0.02 (0.02–0.02)	58.8 (2.9)
B					
(3)	No	Edge	6.0 (5.4–6.6)	0.07 (0.07–0.07)	53.5 (1.9)
		Middle	4.0 (3.6–4.4)	0.10 (0.10–0.11)	59.9 (1.8)
(3)	Yes	Edge	2.8 (2.5–3.1)	0.12 (0.12–0.12)	81.7 (0.3)
		Middle	7.3 (6.7–8.0)	0.05 (0.04–0.05)	74.7 (1.8)
C					
(3)	No	Edge	11.2 (10.3–12.2)	0.23 (0.23–0.23)	50.0 (0.6)
		Middle	13.1 (12.0–14.2)	0.13 (0.13–0.13)	48.3 (1.7)
(3)	Yes	Edge	0.5 (0.4–0.6)	0.00 (0.00–0.00)	81.6 (2.4)
		Middle	0.0 (0.0–0.1)	0.00 (0.00–0.00)	74.8 (2.0)
D					
(3)	No	Edge	3.3 (3.0–3.6)	0.07 (0.07–0.08)	61.8 (2.7)
		Middle	1.3 (1.1–1.5)	0.06 (0.06–0.07)	60.3 (2.0)

decomposition, invertebrate action and uptake by grazers. In October/November 1998 estimates of the 'standing crop' of rabbit faeces was determined for each field by random stratified sampling. This time period was chosen because rabbit numbers were likely to be at their highest [16], the sward at its lowest and consequently grazing livestock, still at pasture as opposed to being overwintered indoors, are at the highest exposure risk.

Forty 0.5 m × 0.5 m plots were assessed by throwing a quadrat: 20 at the edge and 20 in the middle (as defined above). Where a quadrat landed on a latrine (see below) or an area of scrub (gorse or rushes), bare rock or standing water, it was ignored and another thrown. The number of pellets deposited within each quadrat was recorded, as was the average

of five random sward heights measured with a sward stick [17].

The number of latrines (defined as where pellets were encountered at densities >20 pellets/m²) were recorded for ten 1 × 50 m quadrats placed randomly: five at the edge and five in the middle of each field. Three of the six fields on each of farms A, B and C were classed as 'restricted access' and three were not. All three fields on farm D were classed as having no restricted access.

Prevalence of infection in rabbits and infectivity of excreta

Between 14 and 26 rabbits were collected from each farm between August 1998 and December 1999 to

Table 3. Numbers of wild rabbits tested for *M. a. paratuberculosis* on four farms in the east of Scotland. (Numbers in parentheses are positive isolates of *M. a. paratuberculosis* from either lymph node, intestine or faeces samples.)

Farm	Adult males	Juvenile males	Adult females	Juvenile females	Total	%
A	9 (1)	2 (0)	12 (2)	3 (0)	26 (3)	12
B	4 (1)	—	5 (2)	5 (1)	14 (4)	29
C	12 (3)	1 (0)	7 (1)	—	20 (4)	20
D	10 (1)	—	13 (2)	—	23 (3)	13
Total	35 (6)	3 (0)	37 (7)	8 (1)	83 (14)	
%	17	0	19	13		17

Table 4. Isolation of *M. a. paratuberculosis* from wild rabbit urine on four farms in the east of Scotland (numbers in parentheses indicate positive isolates)

Farm	Adult males	Juvenile males	Adult females	Juvenile females	Total tested
A	3 (0)	—	2 (0)	—	5 (0)
B	1 (1)	—	—	1 (0)	2 (1)
C	2 (1)	—	—	—	2 (1)
D	5 (0)	—	3 (0)	—	8 (0)
Total	11 (2)	—	5 (0)	1 (0)	17 (2)

determine the level of infection with paratuberculosis. Rabbits were sexed and classed as adults if weighing more than 1200 g [16]. Smears of lymph node, intestine and faeces were examined for acid-fast bacilli (AFB) by the Ziehl–Neelsen method [1]. Samples with AFB (i.e. ZN+) were cultured and isolates of *Mycobacterium* spp. were identified by a polymerase chain reaction (PCR) to detect the IS900 insertion sequence for *M. a. paratuberculosis* [2].

To investigate the potential for urine to carry the organism, urine from the bladder of rabbits was collected aseptically using a vacutainer blood sampling tube. The urine was centrifuged at 2400 g for 30 min and the deposit cultured. Isolates of *Mycobacterium* spp. were identified by PCR and any mycobacterial isolated confirmed by IS900. Serial dilutions of faeces from infected rabbits were also cultured to determine the level of infection present.

Relative input of bacteria onto grazing pasture

The relative input of *M. a. paratuberculosis* from excreta of cattle sheep and wild rabbits was calculated for the four farms using stocking densities and known disease prevalence, combined with published excretion

rates (for livestock) and data for rabbits calculated from this study (see Table 5 for equations).

Statistical analysis

Raw pellet and latrine data were multiplied (by 4 and 0.02 respectively) to obtain values per m² and normalized by log($x + 1$) transformation [18]. Residual maximum likelihood (REML) [19] was used to estimate the mean values for fixed effects (farm, restricted access and position within field) and their interactions. Genstat REML option [20] was used which approximates standard errors of the differences for the field parameters. The REML model was used to determine the effects of different farms, restricted access and field position on: (1) numbers of rabbit faecal pellets/m²; (2) numbers of latrines/m² and (3) sward heights (mm). Wald tests from the REML routine were used to determine significant differences. The Wald statistic (W) was quoted along with the relevant degrees of freedom and the probability value (compared to a χ^2 distribution) for the fixed effects [20]. Log backtransformed means for faecal pellet and latrine density were presented with 95% confidence limits due to the restriction of backtransforming standard errors [21].

RESULTS

Rates of pellet deposition

The mean levels of rabbit faecal deposition, during the six 2-week periods monitored for the four farms, are presented in Table 1. There was considerable variation between farms with D having the least pellets deposited on grazing pasture. Farm D also had the lowest numbers of rabbits and consequently the least rabbit control conducted on any of the farms. Despite the recorded variation, the overall mean was relatively

Table 5. The relative potential input of *M. a. paratuberculosis* onto grazing pasture based on excretion rates and prevalence for cattle, sheep and rabbits on four farms in the east of Scotland

	Stocking density (per ha)	Defaecations (per day)	Weight of defaecations (g)	Weight of defaecations (g/ha per day)	Prevalence of paratuberculosis (proportion)	'Infectivity' of faeces (c.f.u./g)	Input onto pasture (c.f.u./ha per day)
Cattle	0.26 ^a	11.15 ^c	1770.00 ^e	5131 ^h	0.040 ^k	1.0×10^{8n}	2.1×10^{10q}
Sheep	1.00 ^b	17.33 ^d	82.50 ^f	1430 ⁱ	0.004 ^l	1.09×10^{8o}	6.2×10^{8r}
Rabbits	—	—	0.18 ^g	1234 ^j	0.170 ^m	7.6×10^{5p}	1.7×10^{8s}

^{a,b} mean for farms A, B, C and D; ^{c,d,e,f} means from [22]; ^g mean for 100 rabbit pellets from farms A, B, C and D; ^h ($a \times c \times e$); ⁱ ($b \times d \times f$); ^j ($g \times 7358/\text{ha}$ from this study); ^{k,l} mean for farms A, B, C and D for 1997; ^{m,p} from this study; ⁿ [8]; ^o [9]; ^q ($h \times k \times n$); ^r ($i \times l \times o$); ^s ($j \times m \times p$).

constant over time (Table 1). For all of the farms over the study period the mean number of pellets (\pm S.E.) deposited per 2 weeks/m² was 10.3 ± 3.6 , equivalent to 7357 ± 2571 pellets/ha per day.

'Standing crop' of rabbit faeces

The means for the numbers of pellets, latrines and sward heights for the four farms are presented in Table 2. There was no significant difference between farms ($W=5.1$, D.F.=3, n.s.) or field position – i.e. edge or middle – ($W=0.2$, D.F.=1, n.s.) with respect to the mean numbers of rabbit pellets present. Similarly for the number of latrines encountered there was no significant difference between farms ($W=1.3$, D.F.=3, n.s.) or field position ($W=0.2$, D.F.=1, n.s.). For sward height there was also no significant difference between farms ($W=4.6$, D.F.=3, n.s.) or field position ($W=0.2$, D.F.=1, n.s.). This suggests that for the four farms studied, differences between farms and within fields were not important with respect to the numbers of rabbit faecal pellets likely to be encountered or the height of the sward found.

Restricting rabbit access, however, had a significant effect on the mean numbers of pellets ($W=29.3$, D.F.=1, $P<0.01$) and latrines ($W=9.6$, D.F.=1, $P<0.01$) present and the mean sward height ($W=17.5$, D.F.=1, $P<0.01$), with pellet and latrine numbers lower and sward heights higher in fields with restricted rabbit access. There was a significant interaction between restricted access and farms for the number of pellets deposited ($W=24.2$, D.F.=2, $P<0.01$) and also for the number of latrines encountered ($W=7.2$, D.F.=2, $P<0.05$). Fencing on one farm (C) was more effective at reducing levels of rabbit faecal contamination than on the other farms.

Extrapolating means for fields without restricted access gave a minimum estimate of 61 000 pellets/ha

in the sward plus >20 000 pellets in latrines (where a latrine has >20 pellets) or a total of >81 000 pellets/ha. For fields with restricted access the total was >22 000 pellets/ha (i.e. 14 000 pellets/ha + >8000 in latrines), approximately four times less than fields with no control.

Prevalence of infection in rabbits and infectivity of excreta

M. a. paratuberculosis was isolated from rabbits on all four farms with an overall prevalence of 17% (Table 3). Out of the subsample of 30 rabbits for which urine was available, *M. a. paratuberculosis* was isolated from two rabbits, one each from farms B and D (Table 4).

Serial dilutions to determine the number of colony forming units from infected faeces were completed for 7 samples (2 each from positive rabbits from farms A, B and C and 1 from farm D). The mean number of c.f.u./g from infected rabbit faeces was $7.6 \times 10^5 \pm 5.2 \times 10^5$.

Relative input of bacteria onto grazing pasture

The relative input of *M. a. paratuberculosis* from excreta of cattle sheep and wild rabbits for the four farms are presented in Table 5. It was estimated that cattle, sheep and rabbits contribute 2.1×10^{10} , 6.2×10^8 and 1.6×10^8 c.f.u./ha respectively.

DISCUSSION

Until recently rabbits have been considered largely unimportant as sylvatic reservoirs of livestock diseases [23]. However, the recent detection of bovine viral diarrhoea (BVD) antibodies [24] and isolation *M. a. paratuberculosis* [1, 2] from wild rabbits suggests

that they may play a role in the transmission of disease.

The aim of this study was to quantify the level of rabbit faecal contamination present on grazing pasture on farms where *M. a. paratuberculosis* had previously been isolated from livestock and rabbits. Significant quantities of rabbit faeces were encountered on all four farms studied. The mean deposition rate of 7357 pellets/ha was within the range predicted, based on reported rabbit densities for this part of Scotland and published faecal excretion rates [4–6]. However, this may be an underestimate since sheep continued to graze all fields during the study and may have ingested or trampled on pellets. Due to the accumulation of rabbit faeces over time, the mean ‘standing crop’ encountered was higher – especially for fields classed as having no restricted access (mean of >61 000 pellets/ha).

Previous studies have shown evidence of *M. a. paratuberculosis* infection in 8–100% of rabbits tested on farms infected with paratuberculosis in the east of Scotland [1, 2]. The figures of 13 and 29% reported here are at the lower end of this range. This may in part be due to the inclusion of juvenile rabbits, which had a much lower prevalence of infection (9% as opposed to 18% for adults in the sample tested here – although only a small sample size), but may also be due to the fact that only tissue and faeces from smear positive rabbits were cultured. The isolation of *M. a. paratuberculosis* from rabbit faeces indicates that rabbits on these four farms were infected with paratuberculosis as has been previously demonstrated [1].

This is the first reported isolation of *M. a. paratuberculosis* from urine in wild rabbits. Urine, therefore, may represent another potential mode of transmission onto grazing pasture. For example, badger *Meles meles* urine is regarded as a potential source of transmission of *M. bovis* [25]. However, to date urine has not been implicated in the epidemiology of paratuberculosis partly because urine is known to have an inhibitory effect on the growth of the bacteria in culture media [26]. Furthermore we did not determine the infectivity of rabbit urine in this study and therefore have no data on the potential risk that it may represent. On the other hand *M. a. paratuberculosis* does not grow out with its host and therefore the inhibitory effect may not be relevant. Also, during grazing, swards contaminated by urine may not be avoided to the same extent as faeces [25]. The importance of rabbit urine in the epidemiology of paratuberculosis should be further investigated.

The disease risk to livestock and the efficacy of rabbit control measures

Fields to which rabbit access was restricted had significantly lower numbers of rabbit pellets present – even when the effects of the low numbers of rabbits on farm D (where no restriction was practiced) were taken into account. Furthermore, the higher sward heights associated with restricted rabbit access may further reduce the risk of livestock ingesting pellets. It has been demonstrated that the risks of *M. bovis* transmission to cattle grazing pasture contaminated with badger excreta, are increased as sward height is decreased [25].

Although, the calculations presented in Table 5 directly equate prevalence of infection with excretion rate, this is unlikely as animals excrete at different rates depending on the stage of their infection [10]. Nevertheless from these crude calculations it is apparent that at the livestock density and disease prevalence on the four farms studied here, sheep and cattle contribute approximately 4 and 125 times more organisms/ha per day respectively than rabbits. However, rabbits could potentially contribute 170 million c.f.u./ha per day of *M. a. paratuberculosis* (Table 5) plus additional bacteria through urine. Given that one infected pellet carries a mean of 1.3×10^5 c.f.u., then the ingestion of a small number of infected rabbit pellets could constitute an infective dose. Consequently contamination by rabbit excreta on infected farms may represent a significant disease risk to livestock.

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