

An abattoir-based study of the prevalence of subclinical Johne's disease in adult cattle in south west England

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SUMMARY

The prevalence of subclinical Johne's disease was estimated in adult cattle slaughtered at three major abattoirs in south west England. A polymerase chain reaction (PCR) based on IS900 was used to detect *Mycobacterium paratuberculosis* in intestinal lymph nodes of 1553 cattle. Culture was also carried out on all PCR positive and inconclusive samples. The prevalence of subclinical disease in adult cattle was 3.5% (95% confidence intervals (CI) 2.6–4.7) by PCR and 2.6% (CI 1.8–3.6) by culture. The proportion of the disease in each month ranged from 1.6% (CI 0.2–5.5) in April to 4.6% (CI 2.8–6.9) in November, but the difference was not significant ($P > 0.05$). The proportion of PCR positive lymph nodes in each abattoir ranged from 2.8% (CI 1.6–4.6) to 4.9% (CI 2.9–7.6), this difference was not significant either ($P > 0.05$). The prevalence in young cattle was 2.0% (CI 0.6–4.5). The difference between age groups was not statistically significant ($P > 0.05$).

INTRODUCTION

Paratuberculosis or Johne's disease is a chronic wasting disease of ruminants associated with their immune response to *Mycobacterium paratuberculosis* (*Mycobacterium avium* subsp. *paratuberculosis*) infection. It was recognized 100 years ago [1] and in the 1950s it was believed to be one of the most important diseases of adult cattle in the United Kingdom [2]. The disease still continues to threaten the productivity of ruminants [3–5] and a major limitation to its control is the lack of a reliable diagnostic method of identifying animals with subclinical disease [4, 6].

More recently it has been suggested that *M. paratuberculosis* is associated with Crohn's disease [7–9] and recent developments in molecular biology, in particular the identification of an insertion sequence, IS900 [10], which is specific to *M. para-*

tuberculosis, have facilitated the study of this slow growing organism [11–13].

The frequency of Johne's disease in the UK is unknown. In a recent practice-based survey we estimated the proportion of clinical disease in infected herds to be 1.9% per annum [14]. In other countries, estimates of the prevalence of infection in apparently normal cattle were 0.43% in South Australia [15], 2.3% in Denmark [16] and 1.6–18% in the United States [17–18].

In the present study we investigated the prevalence of subclinical Johne's disease in adult cows in the south west of England, using a polymerase chain reaction (PCR) based on IS900 to identify *M. paratuberculosis*.

MATERIALS AND METHODS

Study design

Three intestinal lymph nodes; the ileocaecal, jejunal and one from the mesenteric lymph chain (Fig. 1), were collected from 1553 adult cull cows in three

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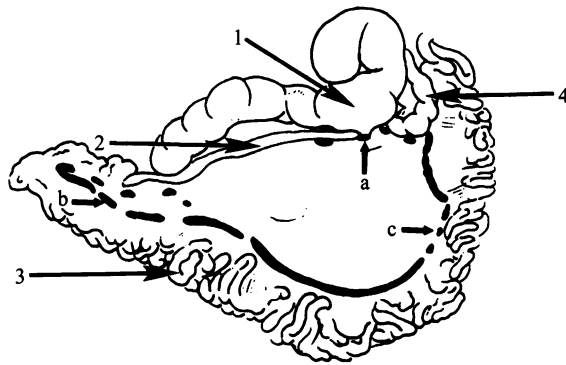


Fig. 1. Diagram of intestines of a cow showing the location of the three lymph nodes collected from each animal. 1, caecum; 2, ileum; 3, jejunum; 4, colon; a, ileo-caecal; b and c, jejunal lymph nodes.

abattoirs in south west England between February and December 1994. These lymph nodes were selected because they drain the terminal ileum and jejunum, harbour the organism at early stages of infection [17, 19] and avoid the problems of faecal cross-contamination.

The sample size required was estimated from an expected prevalence of 5% with 95% level of confidence, and a desired accuracy of 1% [20]. This assumption was made from the results of a recent practice-based survey which we carried out in the same region [14] and from previous studies [21].

Adult cows were sampled in order to determine the frequency of subclinical disease. Johne's disease has a long incubation period (1.5–2 years) [4, 6], and adult cows would be old enough (> 2 years) to have developed clinical disease. The terminal ileum was also checked for gross lesions.

The abattoirs at Keynsham, Langport and Hatherleigh (Fig. 2) were selected from a list of 45, in the counties of Avon, Cornwall, Devon and Somerset in south west England, provided by Meat and Livestock Commission [22]. They were chosen following telephone interviews with the abattoir owners, using the following criteria; a minimum daily throughput of 50 cull cows, their geographical position in the south west region and the distance from our laboratories. The ones chosen were the only ones in the south west which were killing sufficient numbers of cull cows. A further nine abattoirs killed less than 30 cows per week. The rest of the slaughterhouses given in the list just killed young cattle or were small butchers.

In determining the frequency of the visits, the number and pattern of cull cow slaughtering were considered. The total number of cull cows slaughtered

in the south west of England in 1993 was 152000, there was some monthly variation in numbers from 9100 in May to 17400 in October [22]. Because of this difference, weekly visits were carried out between February and May and between September and December 1994 to look for any seasonal variation in the frequency of Johne's disease.

In determining the sampling method at each visit, the number of cull cows from each farm was considered. Auctioneers were contacted and they reported that on average 1–2 cows were sold at markets by individual producers. No attempt was made to determine the farm of origin of the cows but, the market of origin of cattle was obtained from abattoir records.

The number of samples collected each week depended upon the number of cows slaughtered up to a limit of 120. This was the maximum number which could be processed between the visits. When the number of adult cows slaughtered at each visit was small (i.e. less than 50), samples were also collected from young cattle (i.e. heifers and steers) in order to obtain full benefit from each visit. The lymph nodes were cut out with scissors, placed in individual pots, and transferred to the laboratory where the fat was trimmed off. They were kept at -20°C until required.

Sample preparation

A modification of the method reported by Challans and colleagues [23] was used for DNA extraction. Briefly, 1 g of sample was mixed with 5 ml distilled water and mashed up in a stomacher (Seward Medical, model 80) for 2 min at high speed. Five ml of 0.125 M-NaOH was added and the suspension incubated at 56°C for 30 min. One ml Xylene was then added, the suspension shaken for 3–4 min, and left for 20 min to allow the aqueous and organic phases to separate. The organic phase was taken and centrifuged at 11600 g for 10 min. The pellet was washed twice in 0.2 M-NaOH and finally resuspended in $200\ \mu\text{l}$ of 0.2 M-NaOH. An equal volume of 0.1 mm zirconium beads (Biospec Products, Bartlesville, Oklahoma) was added to this suspension and shaken on a mini bead beater (Biospec Product) for $3 \times 90\ \text{s}$, cooling on ice in between. DNA was extracted using saturated phenol followed by polyethylene glycol (PEG) precipitation. After washing the pellets twice with 95% and 70% ethanol respectively, the pellet was dried and resuspended in $50\ \mu\text{l}$ of distilled water.

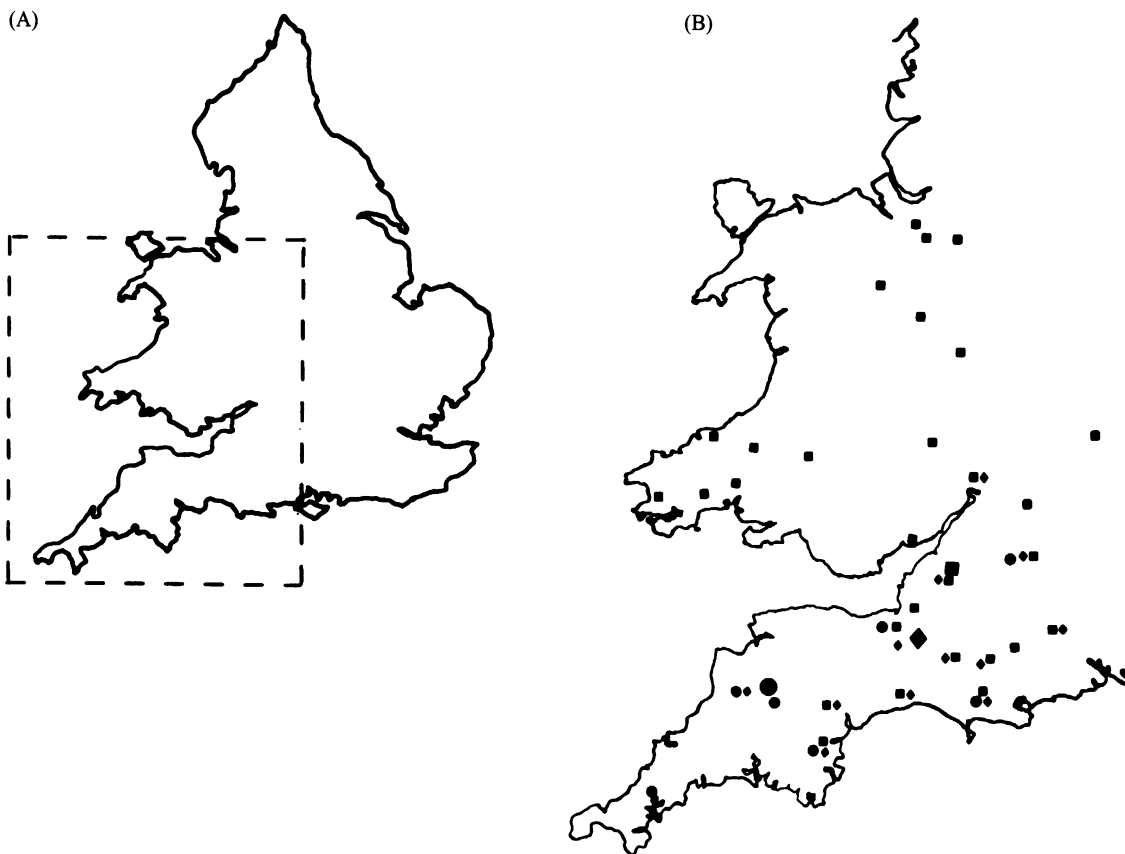


Fig. 2. Map of the UK and the study area (A) showing the location of the abattoirs (■, ◆, ●) (B) and the distribution of markets (■, ◆, ●) which supplied animals to the abattoirs. ■, ■: Keynsham; ◆, ◆: Langport; ●, ●: Hatherleigh.

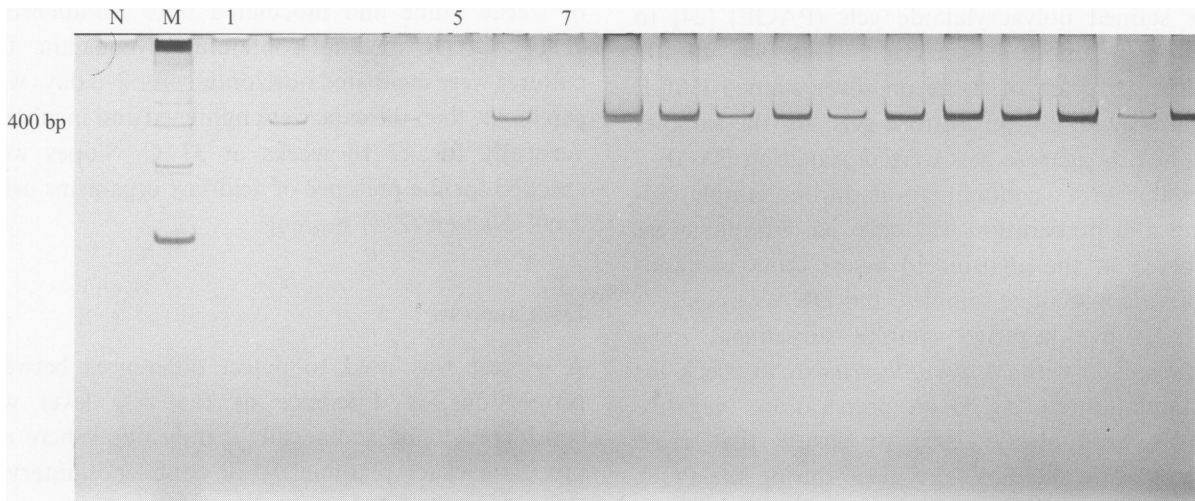


Fig. 3. A silver-stained polyacrylamide gel of PCR amplified products. N, negative control; M, DNA ladder; Lanes 1, 5 and 7, PCR negative samples; and the rest of the Lanes PCR positive lymph node samples with a molecular size of 400 bp.

PCR conditions

The PCR was performed in a thermocycler (Hybaid) in a total reaction volume of 50 μ l containing 5 μ l of 10 \times PCR buffer (100 mM-Tris-HCl, pH 8.3, 500 mM-KCl, 15 mM-MgCl₂, 0.01% (w/v) Gelatin, auto-

claved), 1 μ l of each deoxynucleotide triphosphates, 0.25 μ l (5 U/ μ l) of Taq DNA Polymerase (Perkin Elmer) and 2.5 μ l of primers 90 and 91 derived from IS900 [13] and 5 μ l of template sample DNA. Amplification was obtained with 30 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 60 $^{\circ}$ C for 30 s, and

Table 1. *The proportion of PCR positive lymph nodes from cull cows and young cattle collected at each abattoir*

Abattoir	Cull cows		Young cattle	
	%	CI	%	CI
Keynsham	2.8 (15/530)	(1.6–4.6)	2.2 (1/46)	(0.05–11.5)
Langport	4.9 (18/368)	(2.9–7.6)	2.8 (4/145)	(0.8–6.9)
Hatherleigh	3.3 (13/399)	(1.7–5.5)	—	—
Total	3.5 (46/1297)	(2.6–4.7)	2.0 (5/256)	(0.6–4.5)

synthesis at 72 °C for 1 min with a final extension at 72 °C for 2 min.

The detection limit of the test was evaluated by amplification of DNA extracted from negative tissues spiked with a dilution series (from 1×10^7 to 10) of *M. paratuberculosis* scraped from a positive culture. The number of organisms in the bacterial suspension was estimated spectrophotometrically (LKB Biochrom) by comparison with McFarland standard tubes (Api, Basingstoke) at 550 nm. DNA was then extracted by the procedure described above and 5 µl of samples were amplified in a 50 µl volume of PCR reaction mixture.

The amplified products were initially detected by ethidium bromide stained agarose gels. The method of detecting amplified products was then changed to silver stained polyacrylamide gels (PAGE) [24] to improve the limit of detection. This was 10–100 bacteria per gram of tissue on silver stained PAGE compared with 10^4 on agarose gels. All the samples examined by agarose gels were repeated on PAGE.

Possible cross contamination during sample collection and preparation was checked. Samples were numbered at the abattoir to assess cross contamination of consecutive samples, and DNA was extracted from five negative samples subsequent to a sample spiked with *M. paratuberculosis* to check for contamination during DNA preparation. Negative controls were always used to ensure that cross contamination did not take place during the PCR. Consistently negative results suggested that each step of the assay was free of contamination.

Culture

Tissues from all PCR positive and inconclusive lymph nodes were also submitted for culture. One gram of tissue was mixed with sterile saline in a stomacher. The suspension was transferred into pots containing 45 ml 0.75% cetylpyridinium chloride (CPC) and left

Table 2. *The proportion of PCR positive lymph nodes collected in each month*

Month	Proportion %	CI
Feb	3.0 (4/132)	(0.8–7.6)
Mar	1.8 (3/166)	(0.4–5.2)
Apr	1.6 (2/128)	(0.2–5.5)
May	4.4 (11/250)	(2.2–7.7)
Sep	3.4 (6/178)	(1.2–7.2)
Oct	1.7 (4/238)	(0.5–4.2)
Nov	4.6 (21/461)	(2.8–6.9)

at room temperature overnight (18–24 h). 5–10 ml of supernatant was spun at 3000 g for 15 min, the pellet was then washed with sterile saline and centrifuged again. The final pellet was resuspended in a few drops of sterile saline and inoculated onto Middlebrook 7H11 + OADC medium containing mycobactin. The cultures were incubated horizontally for 2–3 days with cap loose, then the caps were tightened and incubated vertically for 12–16 weeks at 37 °C. Slopes were checked for the presence of acid-fast organisms using Ziehl–Nielsen (ZN) staining.

Data analysis

A χ^2 test was used to detect differences between proportions, a difference at the 5% level was considered to be statistically significant. Where appropriate exact binomial 95% confidence intervals (CI) were calculated [25].

RESULTS

Of the 1553 samples, 1297 were from cull cows and 256 from young cattle. The total number of visits to the abattoirs was 20; 8 to Keynsham, 7 to Langport and 5 to Hatherleigh. The number of samples collected at each visit varied from 40–120.

Positive PCR products with the molecular size of

400 bp (Fig. 3) were obtained from 51 samples, giving a proportion of 3.3% (51/1553) (CI 2.5–4.3) overall. It was 3.5% (46/1297) (CI 2.6–4.7) for cull cows and 2.0% (5/256) (CI 0.6–4.5) for young cattle. This difference between age groups was not statistically significant ($P > 0.05$). The proportion of PCR positive lymph nodes collected from each abattoir is shown in Table 1. The differences between abattoirs were not significant ($P > 0.05$).

The proportion of PCR positive lymph nodes detected each month varied from 1.6% (CI 0.2–5.5) in April to 4.6% (CI 2.8–6.9) in November (Table 2), but this difference was not statistically significant ($P > 0.05$).

A further 22 (1.4%) samples were inconclusive; i.e. at least one positive PCR product was obtained from these samples on agarose gels, but inconsistent results were obtained in PAGE.

Mycobacterial growth in culture was observed in 30 (58.8%) of the 51 PCR positive samples and five (22.7%) of the 22 PCR inconclusive samples. All these cultures were then confirmed as *M. paratuberculosis* by PCR. The same size (400 bp) PCR products were obtained from all 30 samples. When we estimated the proportion of subclinical Johne's disease using the culture positive results only, it was 2.6% (34/1297) (CI 1.8–3.6) in cull cows and 2.3% (35/1553) (CI 1.6–3.1) overall. There were no significant differences between these figures and the ones (3.5% and 3.3% in cull cows and overall, respectively) obtained from PCR results ($P > 0.05$).

DISCUSSION

The aim of the study was to estimate the prevalence of subclinical Johne's disease in adult cattle in the south west of England. There is no reliable test which identifies living subclinical cases, so we used the presence of *M. paratuberculosis* in tissue samples collected at slaughter as a measure of subclinical infection.

In order to avoid collecting samples from animals which were just incubating the disease, the target population was adult cows rather than heifers and steers, because their age would exceed the normal incubation period of 1.5–2 years [4, 6]. However, it is possible that a proportion of the animals sampled would have become clinical cases in time, but the absence of any gross lesions in the intestines of these

animals at slaughter indicated that the development of clinical disease was not imminent.

A PCR based on IS900 was used to detect *M. paratuberculosis* in the lymph node samples and the proportion of positive cases in adult cows was 3.5% (CI 2.6–4.7). The assay is specific for *M. paratuberculosis* [11–13], but it has been reported to have a poor sensitivity when used on clinical specimens such as faeces and tissues because of unknown inhibitors and difficulty in sample preparation [11, 23, 26]. In our hands the limit of detection was 10–100 organisms per gram of tissue. This is similar to that reported for culture [26–28]. PCR is easier to perform and more rapid than culture but detects residual DNA as well as live organisms. For this reason culture was also carried out on all PCR positive and inconclusive samples. Culture also has the advantage of detecting all forms of *M. paratuberculosis*, whereas the use of xylene in the isolation of *M. paratuberculosis* for the PCR test may result in the detection of only hydrophobic bacillary-forms of *M. paratuberculosis*. Therefore, spheroplast forms of the bacteria which will segregate to the aqueous phase may not be detected. Consequently, our method may have underestimated the presence of *M. paratuberculosis* in lymph node samples. Culture of all 1553 samples was beyond the scope and budget of the project. 58.8% (30/51) of PCR positive and 22.7% (5/22) of inconclusive samples were culture positive. The proportion of subclinical infection in cull cows was 2.6% (CI 1.8–3.6), and 2.3% (CI 1.6–3.1) overall by this method.

There are a number of possible reasons for the difference between the number of positive results of culture and PCR. Some of the PCR positive results may represent the presence of dead organisms. The small number and uneven distribution of mycobacteria within the lymph node in subclinical infections may also have resulted in sampling error because different tissue samples were used for culture and PCR.

If we assume that all PCR inconclusive samples were also positive, the proportion for cows would then be estimated as 4.9% (64/1297) (CI 3.8–6.3). A more realistic estimate may be obtained by assuming that the ratio of culture positive:PCR positive samples was the same in each group. The ratio for PCR positive group was 1:1.7; from this the number of PCR positives in the inconclusive samples would be nine, giving a proportion of 4.2% (55/1297) (CI 3.2–5.5) for cull cows. There was a statistically

significant difference between this figure and that of 2.6% (CI 1.8–3.6) obtained by using culture results ($P < 0.05$).

The target population in the study was adult cows, however, the study population consisted of cull cows and a few young cattle. Infertility and mastitis are two of the major health reasons for culling and they have been reported to be associated with subclinical paratuberculosis [29, 30]. For this reason the figures obtained in this study may not represent the adult cow population, but abattoir studies are the only means of investigating subclinical paratuberculosis.

Another factor which may affect the generalization of the results is that, the animals were not sampled randomly. However, attempts were made in the study design to obtain a representative sample by choosing the major abattoirs with strategic geographical positions in the south west and visiting them throughout the year.

The distribution of the markets supplying the abattoirs shows that they received animals not only from widely distributed markets in the south-west, but also from Wales and the Borders (Fig. 2).

We collected all the samples at each abattoir visit because auctioneers reported that 1–2 cows per herd were killed at any time, so the probability of collecting samples from the same herd was small. However, it is possible that animals from the same infected herd may have been sampled at different visits.

There was no significant seasonal variation in the prevalence of subclinical Johne's disease. The highest figure, 4.4% (CI 2.2–7.7), was obtained for May in which the lowest number of cows went through the abattoirs and the lowest 1.7% (CI 0.5–4.2), for October in which the highest number of cows were killed.

In addition to cull cows a small number of samples was also collected from young cattle which could have been incubating the disease. Although the proportion (2.0%) reported here was less than that of 3.5% for cull cows, the difference was not significant ($P > 0.05$).

Previous abattoir-based surveys involving cull cows reported prevalence figures ranging from 6% to 17% [2, 19, 31, 32], with an average figure of 11% [21] for all studies carried out until 1959. The apparent decrease observed in our study may reflect a decrease in the proportion of susceptible breeds (i.e. Channel Islands), or improvements in management and nutrition. Another possibility is that the difference may be due to sampling error.

The suggested association between *M. paratuberculosis* and Crohn's disease in man has rekindled interest in Johne's disease [7–9]. Epidemiological studies of the disease in ruminants are important because they may be potential source of human infection, but more significantly because the molecular markers which enable the strain of *M. paratuberculosis* and genotype of the host immune response to be considered as risk factors for diseases may help in our understanding of the immunopathogenesis of Crohn's disease.

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