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In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: A critical review and meta-analysis

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (*Mptb*) causes Johne's disease in ruminants. Disease control programmes aim to break the faecal–oral cow–calf transmission cycle through hygienic calf rearing and removal of affected cows from the herd, but these programmes do not take account of the potential for congenital infection. The aims of this study were to critically review research on in utero infection, determine the prevalence of fetal infection in cattle through meta-analysis and estimate the incidence of calves infected via the in utero route. About 9% (95% confidence limits 6–14%) of fetuses from subclinically infected cows and 39% (20–60%) from clinically affected cows were infected with *Mptb* ($P < 0.001$). These are underestimates for methodological reasons. The estimated incidence of calf infection derived via the in utero route depends on within-herd prevalence and the ratio of sub-clinical to clinical cases among infected cows. Assuming 80:20 for the latter, estimates of incidence were in the range 0.44–1.2 infected calves per 100 cows per annum in herds with within-herd prevalence of 5%, and 3.5–9.3 calves in herds with 40% prevalence. These estimates were not markedly sensitive to the value chosen for the proportion of clinical cases. In utero transmission of *Mptb* could retard the success of disease control programmes if the opportunities for post natal transmission via colostrum/milk and environmental contamination were able to be controlled. The consequences of fetal infection for the calves so infected are discussed in the context of diagnosis and vaccination together with recommendations for future research.
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Keywords: Paratuberculosis; Intrauterine; Congenital infection; Transmission; Meta-analysis; Review

Introduction

Johne's disease or paratuberculosis occurs globally in ruminants and in cattle it is associated with economic losses due to culling of clinical cases, reduced milk production and the costs of laboratory testing and control measures (Ott et al., 1999). However, potential impact on consumer demand for milk associated with product safety needs to be considered as the causative organism, *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*), may also be a cause of Crohn's disease (Stott et al., 2005; Chamberlin and Naser, 2006). Public health authorities internationally acknowledge that a precautionary approach and further research are warranted.

Most authors agree that the faecal-oral route is the primary mechanism for transmission of *Mptb* and this is reflected in disease control recommendations for cattle (Clarke, 1997). These are similar in most countries and based on removal of clinical cases, identification of sub-clinical cases by objective tests, and hygienic calf rearing (Kennedy and Benedictus, 2001; Benedictus and Kalis, 2003). Compliance with calf rearing recommendations is difficult for some farmers (Wraight et al., 2000), but in any case transmission in utero could limit its effectiveness (Lawrence, 1956; McQueen and Russell, 1979). To the authors' knowledge this topic has never been reviewed formally.

The aims of this study were to critically review published data on extra-intestinal and in utero infection, determine the prevalence of fetal infection in cattle through meta-analysis, estimate the incidence of congenital infection in calves and make recommendations for future research.

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Material and methods

The pathogenesis of *Mptb* infection, evidence for extra-intestinal infection and experimental in utero infection trials were summarised following a literature review. An electronic search was conducted using search terms "in utero", "uterus", "fetus", or "placenta" with the term "paratuberculosis". In addition, a collation of early literature from 1895 (Chiodini, 1992) was searched manually.

A meta-analysis of observational studies of the prevalence of fetal infection in naturally infected cows with Johne's disease was undertaken. Data were included only where the infection had been confirmed in the cow, and to avoid opportunistic investigations with likely extreme prevalence estimates, where more than two fetuses had been examined. Data from studies of high rigour were pooled. These were defined as studies where post mortem methods were specified and indicated awareness of the need to minimise cross contamination of maternal and fetal samples, and where microbiological methods for identification of *Mptb* were described. Studies based solely on identification of *Mptb* using nested polymerase chain reaction (PCR) were excluded due to the risk of false positive outcomes. Studies with more than two fetuses that did not meet the other eligibility criteria are summarised in Table 1. Fetal age was ignored in the pooling of data. Animals with clinical signs consistent with paratuberculosis (for example progressive weight loss and diarrhoea) were classified as clinical cases while infected animals without these signs were classified as sub-clinical cases by the original authors.

Fisher's exact test was used to compare the proportions of infected fetuses from clinically and sub-clinically infected cows, and the odds ratio and its confidence limits were calculated using Prism GraphPad. Exact 95% confidence limits for proportions were calculated using Minitab Statistical Software.

The incidence of calf infection derived via the in utero route was estimated using the upper and lower confidence limits for the prevalence of in utero infection of fetuses in cows with both clinical and sub-clinical infection, estimates of the proportion of infected cows that were clinical cases, and estimates of within-herd prevalence among cows (assuming that each cow produces a live calf each year).

Results

An appraisal of natural in utero transmission of *Mptb* in cattle was informed by review of the pathogenesis of paratuberculosis, extra-intestinal spread of the organism and experimental infections of the bovine reproductive tract. Studies in other species were considered.

Pathogenesis of Mptb infection

Following oral exposure, *Mptb* is taken up by M cells overlying Peyer's patches in the ileum and organisms then move to macrophages in the lamina propria (Momotani et al., 1988). The intestinal lymph nodes become involved and *Mptb* may be found in both locations but not for some weeks after infection (Perez et al., 1996). Chronic, granulomatous enteritis develops where epithelioid cells containing numerous *Mptb* accumulate in the lamina propria and submucosa (multibacillary or lepromatous lesions). However, in some animals *Mptb* may not be numerous in lesions (paucibacillary or tuberculoid lesions).

Cell mediated immune responses initially restrict the organism but later wane, allowing development of the multibacillary form (Clarke, 1997). Serum antibodies are detectable in the later stages of the disease. Cattle may begin to shed *Mptb* in faeces from about 1 year of age and clinical signs of weight loss and diarrhoea occur usually after 2–4 years (Whittington and Sergeant, 2001). Young animals are believed to be most susceptible to infection with an age-based resistance developing. This forms the basis for the hygienic calf rearing techniques that are recommended to control paratuberculosis.

Extra-intestinal spread of Mptb within infected animals

There is a large amount of evidence for extra-intestinal spread of *Mptb* and this occurs most commonly in advanced sub-clinically or clinically affected animals. *Mptb* has been found in extra-intestinal lymph nodes, milk, liver, spleen, semen, testes, epididymis, seminal vesicle and other parenchymous organs of cattle (Pavlik et al., 2000; Barrington et al., 2003; Ayele et al., 2004). There is an extensive literature on the presence of *Mptb* in bovine milk (see, for example, Ellingson et al., 2005). Haematogenous or lymphatic spread are possible routes for movement of the organism to extra-intestinal sites. Indeed, the organism has been found in peripheral blood (Koenig et al., 1993; Barrington et al., 2003; Buergelt et al., 2004; Buergelt and Williams, 2004). There have been similar findings in tissues, blood and milk of sheep, goats, wild ruminants and primates (Morin, 1982; Williams et al., 1983a,b; Reddy et al., 1984; McClure et al., 1987; Gwęzdz et al., 1997; Gwozdz et al., 2000; Naser et al., 2000; Eppleston and Whittington, 2001; Djonne et al., 2003; Lambeth et al., 2004; Juste et al., 2005). As Johne's disease has a systemic component the developing fetus is at risk of infection.

Experimental infection of the bovine reproductive tract

The fate of *Mptb* (5×10^8 colony forming units, cfu, in 5 mL saline) inoculated into the uterus of thirteen 3–4-year-old cows 24 h after service by bull or artificial insemination (AI) was followed at intervals to 28 days after inoculation (Merkal et al., 1982). *Mptb* was isolated from the uterine body and horns 1, 2, 3, 7 and 14 days post inoculation, with one colony also being found in a pelvic lymph node harvested from one cow. The findings indicate the potential for *Mptb* to survive in the uterus and to move to adjacent lymph nodes. Similarly, the intrauterine route was investigated as a means of infection by inoculating three cows with massive doses of the organism (200–400 mg wet weight) at the time of AI (Owen and Thoen, 1983). One cow shed *Mptb* in faeces from 5 months post exposure. This cow was the only one to conceive but aborted at 8 months gestation and *Mptb* was recovered from liver, spleen, mesenteric lymph node and intestine of the fetus. The study was not well designed as the cows may not have been free of Johne's disease when purchased for the trial and the animals cohabitated with three cows given oral doses of *Mptb*.

Mptb may form a close association with the early bovine conceptus. Following the seeding of bovine ova with sus-

pensions of *Mptb* the organism adhered and resisted detachment for up to 10 wash steps (Rohde et al., 1990).

Natural in utero transmission of Mptb in cattle

Studies which were not included in the formal meta-analysis are summarised in Tables 1 and 2. The first report of bovine fetal infection with *Mptb* was made in 1929 (Alexejeff-Goloff, 1929). Acid fast bacilli were visualised in or isolated from the fetal membranes, blood, liver and other tissues of a fetus from a clinically affected cow confirmed with Johne's disease. The paper was abstracted in the Journal of Comparative Pathology in 1935 wherein the editor opined that the finding of intrauterine infection should be regarded as an error of observation.

Serological data from a longitudinal study in various herds indicated a definite familial pattern of infection, with the conclusion that in some cases infection may occur in utero (Hole, 1953). Motivated by cases of Johne's disease of possible congenital origin in Ireland, late term fetuses of three cows were examined and *Mptb* was isolated from one as well as from the uterus of two of the cows (Pearson and McClelland, 1955). Poorly described methods and lack of control over specimen procurement cast doubt on the veracity of these results, but it was probably the first serious attempt to address the question of in utero transmission at the laboratory level. Others followed (Table 1). These early studies were characterised by the isolation of *Mptb* from a wide range of fetal organs and fluids. In addition to fetal infection, organisms resembling *Mptb* were seen in scrapings from maternal caruncles and uterine mucosa from three non-pregnant uteruses from cows with clinical Johne's disease and one uterus was culture positive for *Mptb* (Lawrence, 1956). Ovaries from 1/4 cows with clinical Johne's disease were also culture positive. In another study, fetal membranes of 13/24 fetuses were culture positive (Doyle, 1958).

In many of these studies contamination at slaughter of fetal samples with maternal faeces cannot be excluded, and in one report the organism was isolated from the uterus more often than from a predilection site (Kopecky et al., 1967). Lack of description of post mortem technique precluded the inclusion in the meta-analysis of one large study (Schaaf and Beerwerth, 1960). Of 36 fetuses, 64%

Table 1
Studies that were excluded from meta-analysis of the prevalence of fetal infection in cows naturally infected with *Mptb*

Status of cow	Post mortem method described	Method of detection of *Mptb* described	Number of fetuses (age, months if stated)	Number of infected fetuses	% infected fetus	95% confidence limits	Fetal tissues culture positive	Reference
Subclinical and clinical	No	Yes	3 (8–9)	1	33.3	0.8–90.6	Ileocaecal lymph nodes, intestine	Pearson and McClelland (1955)
Clinical	No	Yes	24	5	20.8	7.1–42.2	Kidney, liver ileocaecal valve, stomach content, spleen	Lawrence (1956)
Clinical	No	Yes	24	9	37.5	18.8–59.4	Spleen, liver, fetal membrane	Doyle (1958)
Subclinical and clinical	No	Yes	36 (2–9)	23	63.9	46.2–79.1	Stomach, intestine, brain, spleen, kidney, lung, heart, liver, testis	Schaaf and Beerwerth (1960)
Not stated	No	Yes	4	0	0	0–60.0	Not applicable	Kopecky et al. (1967)
Clinical	No	Yes (PCR)	3 (2–7)	1	33.3	0.8–90.6	Liver, lung, brain, allantoic fluid	Buergelt and Williams (2003)
Not stated	No	No	9	4	44.4	13.7–78.8	Spleen, kidney, placenta, liver, lung, intestine, brain, abomasal fluid, amniotic fluid	Buergelt and Williams (2003)

Table 2
The results of a trial in which 36 cow and fetus pairs were examined in Europe

Cow						Fetus	
Type of infection	Number	Gross pathology	Histopathology	Serology or allergic reaction	Tissue culture	% infected	95% confidence interval
Clinical	13	Strong positive	Strong positive	Positive	Positive	84.6	54.5–98.0
Subclinical	12	Weak positive	Weak positive	Positive	Positive	58.3	27.6–84.8
Subclinical	11	Negative	Negative	Positive	Positive	45.5	16.7–76.6
Total	36					63.9	46.2–79.1

Data from Schaaf and Beerwerth (1960).

were infected with *Mptb* and there was a trend for greater prevalence of infection in fetuses from cows with more advanced infection (Table 2). Another study was excluded because only nested PCR analysis was used (Buergelt and Williams, 2003).

Meta-analysis of the prevalence of in utero infection in cattle

Only five studies met the criteria for inclusion in the meta-analysis. These were published between 1980 and 2003 and are summarised in Table 3 and Fig. 1. There were 203 fetuses from cows with sub-clinical paratuberculosis and 26 from cows with clinical signs of paratuberculosis. The prevalence of infected fetuses among cows with sub-clinical disease was 9% (95% confidence limits 6–14%). Corresponding figures for cows with clinical disease were 39% (20–60%), and for all infected cows 13% (9–18%) (Table 3, Fig. 1). The risk of fetal infection associated with cows with clinical Johne's disease was significantly greater than that associated with cows with sub-clinical Johne's disease ($P < 0.001$; odds ratio 6.05, 95% confidence interval 2.41–15.20).

A cross sectional study was undertaken in a 102 cow herd in Canada and 37 animals were deemed infected based on tissue culture and/or histopathology; 16 infected animals were histologically negative (de Lisle et al., 1980). Specific details were provided on examination of the uterus, which was removed intact and transported to the laboratory to enable aseptic collection of tissues. Cotyledon, spleen, liver, small intestine and abomasal fluid were examined from 31 fetuses, 19 of which came from infected cows (Table 3). One fetus was culture positive for *Mptb*; its dam, probably a sub-clinical case, was microbiologically and histologically positive. The infection rate was only 3.2% and the authors attributed this to the "minimally infected animals" examined.

In one of the very few studies to examine a large number of cow-fetus pairs with the specific aim of assessing the risk of fetal infection with *Mptb* over 400 animals were tested at an abattoir, including 392 non-randomly sampled clinically normal cows and 15 cows with clinical Johne's disease (Seitz et al., 1989). In this well-designed trial, methods were described in detail, specific instructions were provided to practicing veterinarians who collected some of the samples to prevent contamination of the fetus with maternal com-

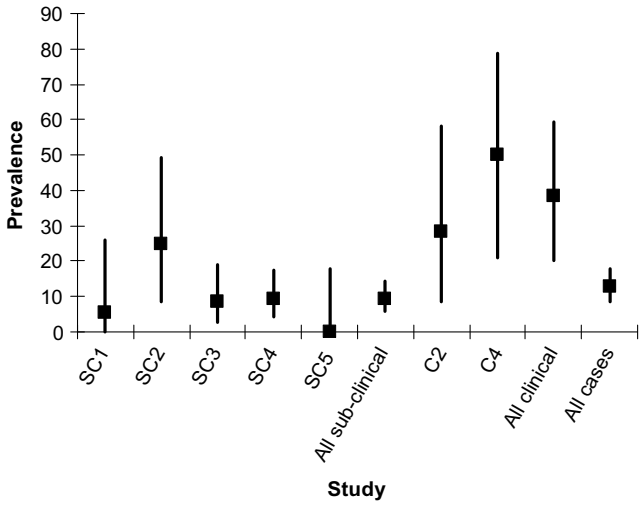

Fig. 1. Percentage of infected fetuses and 95% confidence limits for seven studies included in meta-analysis, with data aggregated for studies of sub-clinical cases (SC1–SC5), clinical cases (C2–C4) and all cases of cow infection. The study identifiers correspond to the data in Table 3.

Table 3
Meta-analysis of the prevalence of fetal infection in cows naturally infected with *Mptb*

Type of infection of cow	Study number	Post mortem method described	Method of detection of *Mptb* described	Number fetuses	Fetal age (as stated)	Number infected fetuses	Fetal tissues culture positive	Reference
Sub-clinical	SC1	Yes	Yes	19	Various	1	Not stated[a]	de Lisle et al. (1980)
	SC2	Yes	Yes	20	All trimesters	5	Various[b]	Seitz et al. (1989)
	SC3	Yes	Yes	58	50–270 days	5	Abdominal viscera, kidney, spleen, ileum, liver, mesenteric lymph node	Sweeney et al. (1992)
	SC4	Yes	Yes	87	Not stated	8	Not stated	Ridge (1993)
	SC5	Yes	Yes	19	35–49 days	0	Not applicable	Kruip et al. (2003)
	Subtotal			203		19		
Clinical	C2	Yes	Yes	14	All trimesters	4	Various[b]	Seitz et al. (1989)
	C4	Yes	Yes	12	Not stated	6	Not stated	Ridge (1993)
	Subtotal			26		10		
All				229		29		

Prevalence estimates are provided in Fig. 1.

[a] A wide range of fetal tissues were cultured – see results.

[b] Undifferentiated abdominal viscera (1/9 fetuses), ileocaecal lymph node (4), ileocaecal valve (3), spleen (3), mesenteric lymph node (1), but not matched to cow category.

ponents, gloves and instruments were changed after handling the dam and the uterus was not touched by the person who removed the fetus from the uterus. Tissue samples removed from carcasses were submitted to the laboratory where maternal tissues were processed separately from fetal tissues, after cleaning the laboratory area. Thus it was unlikely that there had been cross contamination of fetuses from maternal sources. All fetuses from culture negative cows were culture negative. Culture positive fetuses were from all three trimesters of pregnancy.

Another large study specifically addressed sub-clinically infected cows (Sweeney et al., 1992). The authors selected 2–10-year-old cows with positive faecal cultures from 24 herds and divided the animals into groups based on the level of faecal shedding: light (<70 colonies per tube, $n = 30$) or heavy (colonies too numerous to count; $n = 28$) (1 colony per tube = 40–80 cfu/g faeces). The uterus was collected after complete evisceration of the carcass, ligated at the cervix then taken to the laboratory to be opened, except for large fetuses (>200 days old), when the uterus was opened at the abattoir and the fetus transported to the laboratory. Sterile instruments were used to open the fetus and obtain samples for culture. The five culture positive fetuses ranged from 60 to 265 days gestation and were all from heavily shedding cows. All five dams had been seropositive in ELISA. However, this was not predictive of fetal infection as 46/53 cows with a culture negative fetus were also ELISA positive.

In a longitudinal study in 23 herds in Australia, ELISA and/or faecal culture positive cows were culled and 282 were examined at necropsy (Ridge, 1993). Of these, 86% were infected with *Mptb* and fetuses were aseptically collected from 99.

Awareness of reports of *Mptb* infection of fetuses older than 60–70 days led to an intensive study on younger fetuses and reproductive tract products of 16 cows with sub-clinical Johne's disease (Kruip et al., 2003). *Mptb* was not isolated from these sites. The cows were 5 ± 2.8 years-old and were classified as light- to moderate faecal shedders based on identification of 1–100 *Mptb* colonies in faecal samples. Eleven of 16 cows had gross lesions of Johne's disease and histologically 4/11 had multibacillary lesions, four had paucibacillary lesions and three lacked lesions.

Reproductive samples examined microbiologically included uterine contents collected during heat by flushing (one sample per cow), uterine biopsy (five samples per cow collected per vagina on day 8 of the oestrus cycle and homogenised), cumulus–oocyte complex (3–35 per animal collected by transvaginal puncture of follicles), 7 day-old embryos (31 produced in the following cycle by superovulation and AI, collected by flushing) and fetuses 35–49 days old (19, collected after euthanasia of the cow) (Table 3). The authors were aware of the risk of contamination of the genital tract during rectal palpation and provided detailed methods for the collection of samples, in which precautions were described. Notwithstanding the small sample size the authors concluded that there was a low risk of vertical transmission of *Mptb* from cows with moderate degrees of infection, i.e. that the epithelio-chorial placenta does not admit transfer of *Mptb* up to 60 days of gestation (when the cotyledons develop). The lack of uterine infection compared to other studies was rationalised in terms of possible faecal contamination via the vagina in cows with conformational abnormalities, or iatrogenic contamination of the genitalia.

In utero infection of the foetus in species other than cattle

Mptb has been isolated from the fetuses of sheep. In the first report the ewe was in poor body condition but was not regarded as a clinical case (Tamarin and Landau, 1961). There were gross and microscopic changes in the intestine consistent with *Mptb* infection and the organism was isolated. Hepatic lymph nodes from the fetus were culture positive. In subsequent work, reported in scant detail, the authors identified the organism in the mucosa of the uterus of four sheep, of which one was a clinical case and three were CFT reactors. In a more detailed investigation in sheep, necropsies were performed on 142 pregnant 4-year-old ewes from farms in Australia with endemic Johne's disease (Lambeth et al., 2004). Fetal ages ranged from 95 to 149 days. Five of five ewes with clinical Johne's disease had an infected fetus; two ewes had paucibacillary lesions while three had multibacillary lesions. *Mptb* was recovered from the uterus of 4/4 ewes sampled. In addition, one ewe which had no histological lesions and was culture negative from intestinal tissues and associate lymph nodes, was culture positive from the uterus, leading to its classification as a sub-clinical case; this animal had an infected fetus.

Mptb has also been isolated from 1/8 tule elk (*Cervus elaphus nannodes*) fetuses examined during an investigation of Johne's disease ELISA or faecal culture reactors in a herd of clinically normal animals in the USA (Manning et al., 2003b). Fetal infection has also been described in wild red deer in Austria, farmed red deer in New Zealand and a chamois from Austria (Deutz et al., 2005; van Kooten et al., 2006).

Sequelae of bovine foetal infection

There are only two studies related to the outcome of putative in utero infection. In the first report, published in 1935, a 1-week-old bull calf was studied when its dam presented with clinical Johne's disease (Dunkin, 1935). It developed clinical paratuberculosis at 3.5 years of age and the author presumed in utero infection as it had been delivered manually, was not allowed to make contact with the ground or any unwashed part of the cow's exterior, was raised in isolation then fostered to a cow from a Johne's disease free herd. The calf reacted to a Johnin skin test on several occasions between 3 and 12 months of age and at slaughter *Mptb* was found in faeces, paratuberculous

lesions were found in the rectum, ileum and ileo-caecal valve, direct smears of mesenteric lymph node and intestine contained acid fast bacilli and there was no evidence of tuberculosis to account for past skin test reactions.

The other study was published almost 70 years later (Manning et al., 2003a). A cow seroconverted 14 months after acquisition, when it was 6 months pregnant. The calf was delivered by caesarean, but by this time the cow had clinical signs and gross pathology consistent with Johne's disease; histopathology confirmed multibacillary lesions, and tissue cultures were positive for *Mptb*. Details of hygienic calf rearing were provided and the authors stated that the probability of horizontal transmission of *Mptb* was very low. There were no clinical signs of Johne's disease when the calf was slaughtered when 2-years-old but there were typical gross pathological signs of Johne's disease and there were paucibacillary lesions containing some acid fast bacilli. While not absolutely certain, this report suggests the consequences of in utero infection with *Mptb* include development of sub-clinical Johne's disease, histological lesions and therefore, possible progression of lesions and faecal shedding of the bacterium. Thus in utero infection may result in typical Johne's disease expression.

Incidence of calf infection acquired in utero

The incidence of calves infected as fetuses depends on the ratio of sub-clinical cases to clinical cases among infected cows, and on within-herd prevalence. There are no reliable estimates of the former, but common scientific opinion is that sub-clinical cases are dominant (Whitlock and Buergelt, 1996). Estimates of within-herd true prevalence are surprisingly uncommon. In a recent study in the USA, up to 15.5% of cows in 35 herds (average size 450 cows) from 21 states tested positive in a serum ELISA (Lombard et al., 2006). True prevalence would be higher. Data from other serological studies suggest that within-herd true prevalence can range from very low (close to zero) to about 80%, with most estimates being in the range 1–15% (McNab et al., 1991; Collins et al., 1994; Whitlock and Buergelt, 1996; Ott et al., 1999; Muskens et al., 2000; Nielsen et al., 2007; Van Schaik et al., 2003; Jubb and Galvin, 2004).

For a herd where 5% of cows are infected, between 0.44 and 1.2 infected calves per 100 cows per annum would be expected (Table 4). Corresponding figures for within-herd prevalence of 40% are 3.5–9.3 infected calves per 100 cows

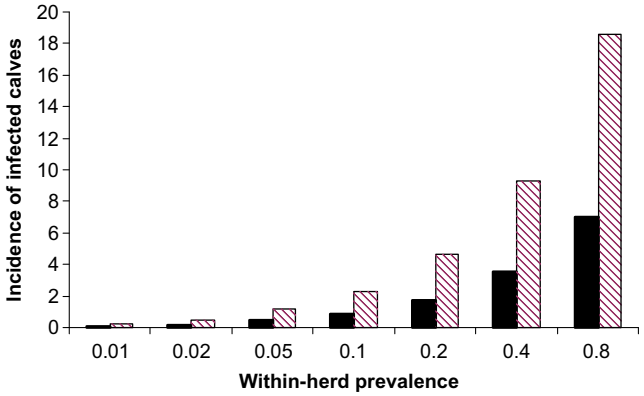

Fig. 2. Estimated incidence of calves infected via the in utero route expressed as the number of infected calves per 100 cows per year for points within the reported range of within-herd prevalence. Incidence was estimated using 95% confidence limits (lower, black bars; upper, striped bars) assuming that 20% of cow infections were clinical.

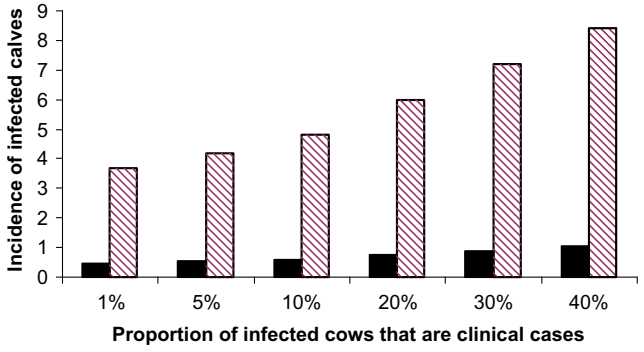

Fig. 3. The effect of the proportion of infected cows that are clinical cases on the estimate of incidence of calves infected via the in utero route. The data were based on mean fetal infection rates for sub-clinically infected cows (9%) and clinically infected cows (39%) for two levels of within-herd prevalence: 5% (black bars); 40% (striped bars).

Table 4
Number of calves with in utero derived *Mptb* infection expected per 100 cows per annum. An example is shown for a 100 cow herd with 5% within-herd prevalence

Cow data	% cows	Number of cows	% calves infected in utero[b]	Number of calves infected in utero
With calves		100		
Infected with *Mptb*[a]	5			
Clinical cases as a proportion of all infected[a]	20			
Infected		5		
Sub-clinical infection		4	6–14	0.24–0.56
Clinical infection		1	20–60	0.20–0.60
Total				0.44–1.16

Values for all levels of prevalence reported to date are provided in Fig. 2.
[a] See methods for sources of these estimates.
[b] Upper and lower 95% confidence limits for each cow-class – see Fig. 1.

per annum and values for other levels of prevalence can be determined from Fig. 2. These estimates were not markedly affected by the value chosen for the proportion of infected cows that were clinical cases (Fig. 3). For example, over an extreme range of values (1–40% of infected cows being clinical cases) the estimated incidence of infected calves ranged from 0.47 to 1.05 per 100 cows per annum for a herd with 5% within-herd prevalence.

Discussion

The pathogenic mycobacteria have complex host-parasite relationships and in general have more than one mechanism of transmission between animals. While faecal–oral transmission of *Mptb* is likely to be the dominant means of perpetuation of Johne's disease in livestock, it is unlikely to be the only means. It is widely acknowledged that *Mptb* is shed in bovine milk (Ellingson et al., 2005). Both faecal–oral and trans-mammary transmission risk is reduced through hygienic calf rearing in which cows and calves are separated soon after birth and calves are reared on milk replacer in an environment that has not been contaminated with *Mptb*.

In utero infection with *Mptb* was first raised as a potential means of familial transmission of the organism in cattle in 1929, with many authors since then commenting on how it would affect control programmes that are based on hygienic calf rearing. It is remarkable then that so little research has been undertaken in the modern era when control programmes have been initiated in many developed countries for cattle, sheep, deer and other species. Difficulties experienced by farmers in compliance with hygienic calf rearing recommendations (Wraight et al., 2000) may have overshadowed concerns about alternative transmission routes.

There are three procedural factors that influence estimates of the rate of fetal infection in cows with Johne's disease: iatrogenic contamination of fetal samples with maternal faeces, the method of culture of the organism and the method of identification of the organism. These factors were assessed critically in each publication prior to meta-analysis.

It was difficult to draw strong conclusions from the early literature on in utero transmission of *Mptb* because there was lack of evidence of adequate awareness of cross contamination of the fetus with faeces of the dam (Table 1). Thus the work of researchers in the 1920s, 1950s and 1960s was of general interest only. A possible exception was a detailed study published in German in which a very high proportion (85%) of fetuses from clinically affected cows were infected, with a high proportion (52%) of fetuses from subclinically infected cows also infected (Schaaf and Beerwerth, 1960). All later studies identified a lower prevalence of fetal infection in both categories of infected cows. As details of the post mortem technique used in the German study were not provided, it was excluded from the meta-analysis, but the findings might have been valid.

All culture methods for *Mptb* involve decontamination in one or more disinfectants that kill a large proportion of *Mptb* cells. For example one commonly-used protocol destroys more than 99% of the viable organisms in a sample (Reddacliff et al., 2003). Furthermore, all of the studies shown in Tables 1–3 used culture on solid media to isolate *Mptb*. These media have lower analytical sensitivity than BACTEC radiometric culture (Eamens et al., 2000) and other things being equal will detect fewer fetuses with low numbers of *Mptb* present. For these reasons the prevalence of fetal infection with *Mptb* would likely be higher than that reported in many of the papers in the literature.

Identification of *Mptb* was problematical before discovery that the organism was mycobactin dependent, and before the era of molecular biology. Microbiologists once relied on guinea pig inoculation to differentiate acid fast bacilli – if disease did not develop the organisms were deemed not to be *M. tuberculosis* (or *M. bovis*) (Tamarin and Landau, 1961; Wilson and Miles, 1975). Confirmation of *Mptb* relied on inoculation of lambs or calves to demonstrate potential to cause lesions of Johne's disease, but this was rarely done due to cost and time. Thus in many cases *Mptb* was identified based only on slow growth, morphology in stained smears and tissue predilection in the host of origin, that is, a presumptive identification. These factors might lead to overestimation of the prevalence of fetal infection with *Mptb* if mycobacteria other than *Mptb* can cause fetal infection. Even in the studies used in meta-analysis in Table 3, identification of *Mptb* may not be absolutely certain because it was rare for both cultural and specific molecular criteria to be applied until the late 1990s. However, the impact is considered to be theoretical as there are unlikely to be other mycobacterial species involved in cattle where infection leads to clinical and pathological signs consistent with Johne's disease.

The findings from the meta-analysis signified substantial risk of in utero transmission of *Mptb* from cows with *Mptb* infection, including cows with sub-clinical or mild infections. The incidence of calves infected as fetuses could be significant on some farms. However, the estimates made in this study may not have universal validity as the ratio of sub-clinical to clinical cases is uncertain, even though expert opinion is that sub-clinical cases predominate (Whitlock and Buergelt, 1996), and estimates of within-herd true prevalence were also uncommon, despite decades of research on paratuberculosis in cattle.

Within-herd prevalence would be affected by the duration of herd infection, whether or not control measures such as test and cull were practiced in the herd, assumptions about test sensitivity and specificity and other factors. Regardless, such estimates tend to be underestimates because of the long incubation period of paratuberculosis and the dependence of test sensitivity on stage of infection (Whittington and Sergeant, 2001). For this reason estimates were provided on incidence of calf infection over a wide range of within-herd prevalence. Even at a level of within-herd prevalence as low as 5% the estimated incidence of calf

infection in large herds could be substantial (0.44–1.2 infected calves/100 cows/per annum). However, the measurable impacts of in utero infection on inter-generational disease transmission are confounded by widespread opportunities for post natal transmission via milk and the contaminated environment on most farms. In utero transmission of *Mptb* could retard the success of disease control programmes if the opportunities for post natal transmission were able to be controlled.

Infection of the fetus may be present at any stage of gestation except perhaps <60 days (Kruip et al., 2003) and involves a wide range of fetal organs, the fetal membranes and the structural elements of the placenta – the cotyledons. However, there were inconsistencies in reporting between studies. In general not every tissue was examined from every fetus and aggregate results were expressed at the level of the fetus rather than tissue in most studies. Thus it is not possible to determine from the literature which fetal tissues represent the best option for culture in future studies, but many of the abdominal viscera appear suitable.

The consequences of in utero infection with *Mptb* for the calves so infected and for subsequent control of Johne's disease in herds are unknown (Seitz et al., 1989; Sweeney et al., 1992). Infection with *Mptb* is probably not lethal to the fetus in most cases, except where there has been massive exposure (Owen and Thoen, 1983). Evidence for this includes the isolation of *Mptb* from fetuses in each trimester of gestation and at term. Further evidence is the observation that infertility due to early embryonic death and abortion are not considered to be signs of endemic Johne's disease in cattle or other species. There are only two reports where putative in utero infection has been followed through to clinical outcome (one clinical case, one subclinical case). There have been no unbiased longitudinal studies to follow the outcome in a cohort of animals exposed and infected in utero. Such a study would be difficult. The consequences of fetal infection with *Mptb* could include (1) progressive infection, manifest as faecal shedding then development of clinical disease (in herds practicing hygienic calf rearing it would manifest as apparent failure of the hygienic calf rearing program); (2) immune tolerance with or without persistent infection (this may depend on the time of infection in relation to the development of immunocompetence in the fetus, and may manifest as lack of lesion development due to immunotolerance, failure to react in diagnostic tests, failure to respond to vaccination and possible shedding); (3) recovery and elimination of the organism.

Prediction of fetal infection based on ante-mortem examination of an individual cow is not currently possible. However, cows with clinical Johne's disease have a relatively high risk of delivering a calf with *Mptb* infection acquired in utero and are more than four times as likely to do so as sub-clinically infected cows. Faecal shedding is a lesser predictor, while ELISA status of the cow appears to be an unreliable indicator of fetal infection. There have been no studies to examine the use of fetal biopsy (membrane or allantoic fluid) as a tool to identify fetal infection, although it has been suggested (Buergelt and Williams, 2003).

The mechanism(s) of infection of the fetus is unknown and cannot be inferred from the published studies. It may involve haematogenous spread to the tissue of the pregnant uterus, followed by colonisation of or movement through the maternal caruncle and fetal allantochorian. *Mptb* has been detected in maternal blood and both placental tissues. It is unknown whether the organism is trafficking within macrophages or is "free". Another possibility is that the organism gains access to the uterus via the vulva, associated with poor vulval conformation and faecal shedding (Kruip et al., 2003). If this was the case it might be prevented by negative selection based on perineal conformation.

In utero inoculation of *Mptb* during natural mating or AI remains a theoretical avenue for infection of the fetus. The organism may originate within the semen or be derived from faeces that may be carried into the uterus on the penis or pipette. There are reports of *Mptb* in semen of both cattle and sheep (Eppleston and Whittington, 2001; Ayele et al., 2004). *Mptb* can survive for some days after inoculation into the uterus and may spread to local lymph nodes (Merkal et al., 1982). Unlike the other routes of in utero contamination, AI risk can be managed through quality assurance programmes in artificial breeding centres and hygienic insemination technique (Wentink et al., 2000).

Conclusions

Research approaches to better understand and intervene in the process of in utero infection are likely to be difficult and expensive. The needs are to understand (1) the mechanism of access of *Mptb* to the uterus, particularly the relative importance of haematogenous spread, direct extension via the placental tissues, and per cervical infection with faecal-derived organisms; (2) whether the immune status of the cow influences in utero transmission to the fetus, and in particular whether vaccination of the cow, which is known not to prevent infection, would limit extra-intestinal spread of *Mptb*; (3) the consequences of in utero infection of the fetus in relation to immune status, efficacy of calfhood vaccination, application of diagnostic tests and clinical outcome. Basic knowledge of the immune response in calves already infected by the time of birth is required. If immune tolerance occurs, these calves may turn out to be "non-responders" to vaccine and succumb to clinical disease despite vaccination.

Longitudinal studies are required. The prevalence of in utero infection would be high enough to be able to include a significant number of infected calves collected from known infected cows (about nine infected calves for every 100 collected). Risk factors could be studied in the cows. Calves would need to be reared in such a way to prevent

horizontal transmission and for long enough to measure meaningful outcomes.

In order to prevent vertical transmission of *Mptb* pending availability of additional data, it is recommended that all direct maternal relatives and progeny of cows with Johne's disease confirmed histologically or microbiologically (which includes sub-clinically infected cows) be removed from a herd. This reiterates suggestions made in earlier studies (Schaaf and Beerwerth, 1960; Ridge, 1993).

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