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Mycobacterium avium subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease

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ABSTRACT

Blood, liver, kidney, lymph nodes and muscle tissue were obtained from the carcasses of five cows with advanced Johne's disease. Samples from the raw tissues, from cooked muscle tissues and from cooked hamburger patties that contained chopped mesenteric lymph nodes were collected aseptically. Each sample was divided into two portions, one of which was decontaminated. Both portions were homogenized. Homogenates were spread on selective agar for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* (Map) and inoculated into a Map growth medium with the organism being detected in the cultures by PCR procedures and Ziehl–Neelsen staining. Map were recovered at numbers $>10^3$ cfu/g from 7 of 15 liver and mesenteric and ileocaecal lymph node samples; and at lesser numbers from 5 of 15 kidney and superficial inguinal and prescapular lymph node samples. The numbers recovered from decontaminated and not decontaminated portions of each sample were generally similar. Map was recovered from 1 and detected in 6 of 50 not decontaminated portions of samples of raw, chilled or frozen meat; and detected in 1 of 15 not decontaminated samples of meat cooked to 61 °C, and in 1 of 40 samples of meat cooked to ≥70 °C. Map was detected in 2 of 4 samples of mesenteric lymph nodes cooked to 61 °C, but not in samples cooked to ≥70 °C. The findings indicate that Map may be present in meat from infected animals at low numbers, but that any such organisms are likely to be inactivated when meat is cooked to a well done condition.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (Map) causes Johne's disease, a chronic enteritis, in cattle and other ruminants. Cattle are commonly infected while young, but clinical symptoms of weight loss and diarrhoea usually appear only when animals are two years or older (Whitlock and Buergelt, 1996), and many infected older animals are asymptomatic (McKenna et al., 2004). In infected animals, gut tissues and lymph nodes associated with the gut become heavily colonized by Map, and the epithelium develops a characteristic corrugated appearance (Cocito et al., 1994). In advanced cases of the disease Map can be widely disseminated in other organs and lymph nodes (Ayele et al., 2004).

In humans with Crohn's disease, a chronic inflammatory condition of the gut, the gut wall develops an appearance similar to that found in animals with Johne's disease. Therefore it has long been suspected that Map is involved in Crohn's as well as in Johne's disease. However, gut tissues from Crohn's disease patients do not contain large numbers of acid-fast Map. There have been reports of small numbers of non-acid-fast forms of the organism in such tissues (Chacon et al., 2004), but those findings are not generally regarded as conclusive for establishing Map as the cause of Crohn's disease. Thus, what relationship there may be between Map and Crohn's disease remains uncertain (Feller et al., 2007; Selby et al., 2007; Shanahan and O'Mahony, 2005). Even so, minimizing the exposure of humans to Map is considered desirable, as a precautionary measure, and some regulatory authorities are taking action on the matter (Gould et al., 2005).

As carcasses are inevitably contaminated with bacteria of faecal origin during carcass dressing (Gill, 2005), contamination of beef with Map from the faeces of animals that were shedding the organism must be expected. The organism might also be present in some tissues used for meat that are taken from the carcasses of cattle in which the organism was disseminated, but there are as yet few reported studies of such matters. Map DNA was detected in swab samples from the carcasses of both young and older cattle, but in amounts that suggested it was unlikely that the fastidious and slow growing organism could be recovered from such samples even if viable Map were present (Meadus et al., 2008). The reported failure to detect Map DNA in samples from 500 portions of retail ground beef would support that suggestion (Jaravanta et al., 2007). However, in a study of

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tissues from 21 cattle with disseminated Map infections, Map were cultured from muscle associated lymph nodes from a few of the animals although all samples of muscle tissue were Map-negative (Antognoli et al., 2008).

Although the available information suggests that even beef from older cattle may usually be contaminated with few Map only, the published data provide no indication of the extent to which some beef might be contaminated by Map from infected lymph nodes. Moreover, the extent to which Map in beef tissues are likely to be inactivated by cooking is uncertain, given that the organism has been reported to sometimes survive usual pasteurizing of milk (Grant, 2005). Therefore, for better understanding of the extent to which consumers might be exposed to Map in beef, raw and cooked tissues from five cows with advanced Johne's disease were examined for the organism.

2. Materials and methods

2.1. Collection and storage of control samples

Control samples were prepared before samples were obtained from Map infected cows. Five strip loins from fed beef cattle less than 2 years old were obtained from a large commercial beef packing plant. The strip loins were selected at random from the product vacuum packaged on the day they were collected. The strip loins were cut into steaks about 3 cm thick which were not cooked or were cooked to an end point central temperature of 72 °C. The latter steaks were cooked on a hot plate operated at 170 ± 2 °C, and were turned every 30 s, with the temperatures at the centres being monitored using a thermocouple sensor, as previously described (Gill and McGinnis, 2004).

Raw and cooked steaks were sampled similarly, with cooked steaks being sampled immediately after the completion of cooking. A block of meat that measured approximately 6 × 6 × 3 cm was excised from the centre of each steak. The block of meat was immersed in boiling water for 1 min, and then the surfaces were dried and the two larger surfaces were seared to a depth of 3 mm by means of a heated steel plate fitted with a handle. A core measuring approximately 4 × 4 × 2.5 cm was cut from but retained in the block. While still in place in the block, the core was cut into strips which were dispensed into plastic tubes to obtain 5 g samples of meat. Sterile equipment was used at all stages of steak sampling, the procedure for which has been previously described (Gill and McGinnis, 2004).

When control steak samples were prepared a sample from each of a raw and a cooked steak from each strip loin was pummeled with 45 ml of 0.1% (wt/vol) peptone water using a stomacher. Two 10 ml portions of the stomacher fluid were each mixed with 2 ml of papain solution (EZ enzyme; Oxoid, Mississauga, Ontario, Canada) and the mixtures were incubated at 25 °C for 30 min. All of each mixture was then filtered through a hydrophobic grid membrane filter (HGMF; Oxoid). Each filter was placed on a plate of tryptone soy fast green agar (TSFGA; Oxoid) which was incubated at 25 °C for 3 days before filters were examined for the presence of colonies.

In addition, ten blocks of meat that each measured about 5 × 5 × 5 cm were cut from commercial strip loins obtained as before. Each block of meat was placed in boiling water for 1 min, and dried using sterile sponges; then all faces of the block were seared to a depth of 3 mm by pressing the block on a steel sheet heated on a gas ring. The blocks were ground through a sterile grinder head using plates with first 8 mm then with 3 mm holes, with the product from the ten blocks being mixed by hand while wearing sterile gloves after each stage of grinding. Two 5 g samples of the ground product from each strip loin were pummeled with diluent using a stomacher, and portions of the stomacher fluid were filtered for enumeration of total aerobic bacteria, as before.

The remaining product was used to form twelve patties by hand, while wearing sterile gloves. Each patty was approximately 10 cm in diameter and weighed approximately 100 g. Four patties were cooked to 72 °C at their centres, and 5 g samples of the cooked product were collected from the centre of each patty.

The eight remaining patties were used to prepare control samples containing lymphatic tissue. Mesenteric lymph nodes that had been obtained from the viscera of fed beef cattle at the beef packing plant were each dipped in boiling water for 20 s. The lymph nodes were then chopped in a sterile blender operated at low speed. A 5 g portion of the chopped lymph nodes was spread over an area about 4 cm in diameter at the centre of each of four of the patties. A second patty was placed on each of the patties spread with lymphatic tissue and the composite patties were cooked to 72 °C at their centres. After cooking, the portion spread with lymphatic tissue was cut from the centre of each patty and 5 g samples of those portions were collected.

All the control samples, each in a separate plastic tube, were frozen in liquid nitrogen then stored at −18 °C.

2.2. Collection and storage of samples from Map infected cows

Five cows identified by attending veterinarians as having overt symptoms of advanced Johne's disease, and found to be shedding Map in their faeces were obtained from dairy herds. On each day that samples were collected, one cow was slaughtered at a research abattoir. Before slaughter, samples of blood were collected aseptically into whole blood collection tubes (VWR International, Mississauga, Ontario, Canada); and a sample of faeces was collected from the rectum except, inadvertently, from cow 4. When the viscera had been removed from the carcass, a portion of the liver and a kidney, and whole or parts of each of popliteal, prescapular, superficial inguinal, ileocaecal and mesenteric lymph nodes were collected in a separate plastic bag. The *semimembranosus*, *semitendinosus*, *biceps femoralis*, *gluteus medeus* and *longissimus dorsi* muscles were then removed from the carcass and placed in plastic bags. Sterile gloves were worn and sterile equipment was used for the collection of all tissues. All muscles were stored overnight at 4 °C. The fecal samples and all other tissues, except about 100 g of the mesenteric lymph nodes from all carcasses except that of cow 1, were stored on ice. The 100 g of mesenteric lymph nodes from each of the other four carcasses was dispensed in approximately equal amounts into five sterile plastic tubes which were capped and frozen in liquid nitrogen.

Five steaks, each about 3 cm thick were cut from each of the *semimembranosus* (inside round), *semitendinosus* (eye of round) and *biceps femoralis* (outside round) muscles. A steak from each muscle was assigned to not being treated, cooked to a temperature at the centre of 61, 70, or 75 °C, or freezing. Steaks were cooked as were the steaks used for the preparation of control samples, or steaks were frozen individually in sterile plastic boxes stored at −18 °C. Samples were obtained from each steak as were the control samples.

The tissues remaining from the muscles used for the preparation of steaks, and the *gluteus medius* and *longissimus dorsi* muscles were ground as was the meat from commercial strip loins. Ten 5 g samples of the raw ground product from each carcass were collected into plastic tubes. The remaining product was used to form six patties by hand, as before. Two of the patties were cooked to 72 °C at their centres, two were frozen in liquid nitrogen, and two were frozen at −18 °C as were the steaks. After cooking or thawing 5 g samples were obtained from each patty, as before.

The tubes containing portions of mesenteric lymph nodes collected from each of four of the carcasses and frozen for storage were immersed in water of 37 °C. The thawed lymph nodes from each portion were then dipped in boiling water and chopped as were the mesenteric lymph nodes from commercial carcasses. Six 5 g portions of the chopped lymph nodes were collected, then six 5 g portions were each spread over an area 4 cm in diameter at the centre of a patty prepared from commercial strip loins, as before. A second patty of the same sort was placed on each of the six patties spread with lymphatic tissue. Two of the composite patties were cooked to each of

61, 70 and 75 °C at their centres. After cooking, the portion spread with lymphatic tissue was cut from each patty and four 5 g samples were collected from that portion.

2.3. Testing samples for Map

Samples of raw and cooked meat from Map infected cows were held on ice before some of the samples were transported to a second laboratory within 24 h of being collected. All the remaining samples were frozen in liquid nitrogen and stored at −18 °C. The chilled samples from each carcass received at the second laboratory on each of five days were one sample of faeces, except for faeces from cow 4; one sample of each of blood, kidney and liver; one sample of each of the popliteal, prescapular, superficial inguinal, ileocaecal and mesenteric lymph nodes; a set of samples from each of the inside round, outside round and eye of round muscles, with each set being composed of one sample from each of a steak that was raw or cooked to 61, 70 or 75 °C; and one sample of raw and two of cooked ground meat. With each of those groups of chilled samples the laboratory also received three samples of each of raw and cooked intact muscles and a sample of each of raw and cooked ground muscle control samples that had been thawed before they were dispatched to the second laboratory.

On each of five other days, the second laboratory received samples that had been frozen and thawed, from a single cow carcass. Those groups of samples were from a steak frozen at −18 °C from each of the inside round, outside round and eye of round cuts, and two samples of each of ground meat frozen at −18 °C or with liquid nitrogen.

On each of four further days the second laboratory received a sample of frozen and thawed, raw, chopped mesenteric lymph nodes from a single carcass; and samples of paired hamburger patties on one of which the chopped lymph nodes had been spread and which had been cooked to 61, 70 or 75 °C. In addition the laboratory received on each of those days, a sample from paired hamburger patties that had been spread with chopped lymph nodes from a commercial carcass and cooked to 72 °C; and on two days a sample of raw chopped lymph nodes from commercial carcasses.

Each 5 g sample of tissue was placed in a sterile stomacher bag, and broken up by pounding flat before it was pummeled in a stomacher with 45 ml of sterile 0.9% (wt/vol) saline. The stomacher fluid was poured from the bag, through a pad of sterile medical gauze, into a 50 ml centrifuge tube. Each of two slants of Herrold's egg yolk agar (HEYA) supplemented with mycobactin J at 2 mg/l and antibiotics (VAN™; vancomycin, amphotericin B and naladixic acid), manufactured by BBL (Becton Dickinson, Sparks, MD, USA), was inoculated with 250 µl of the filtrate. A 500 µl portion of the filtrate was inoculated into a mycobacteria growth indicator tube (MGIT™) containing BACTEC™ MGIT™ Para TB medium (Becton Dickinson; modified Middlebrook 7H9 broth with mycobactin J), and BACTEC™ MGIT Para TB supplement (Becton Dickinson; bovine albumin, catalase, casein and oleic acid). The remaining stomacher fluid was centrifuged at 3000×*g* for 30 min, and the supernatant was discarded. The pellet was resuspended in 0.9% saline to obtain a suspension of 6 ml final volume. An equal volume of a freshly prepared decontaminating solution composed of 2% (wt/vol) NaOH, 0.5% (wt/vol) N-acetyl-L-cysteine and 2.9% (wt/vol) trisodium citrate dihydrate was added to the suspension while it was swirled. After holding at room temperature for 15 min, 38 ml of 134 mM, pH 6.8 phosphate buffer was added, and the mixture was centrifuged at 3000×*g* for 30 min. The supernatant was discarded and the pellet was resuspended in 2 ml of Middlebrook 7H9 broth (Difco) supplemented with Middlebrook OADC (oleic acid, bovine albumin, dextrose and catalase) enrichment (Difco) and mycobactin J (Allied Monitor, Fayette, MO, USA) at 2 mg/l. A 250 ml portion of the suspension was inoculated onto each of two HEYA slants and into a tube of supplemented MGIT™ Para TB medium.

All slants and tubes of inoculated media were incubated at 37 °C for up to 20 weeks. HEYA slants were examined weekly for the presence of colonies. Colonies that initially were buff-coloured, smooth and domed were presumed to be Map. Colonies were confirmed as Map by Ziehl–Neelsen (ZN) staining for detection of acid fast cells, and by a direct PCR procedure for detection of the *IS900* sequence. The levels of detection of colonies on HEYA slants were 40 and 12 cfu/g for not decontaminated and decontaminated portions of samples, respectively.

MGIT cultures were tested weekly for fluorescence when exposed to ultraviolet light. Smears of fluorescent cultures on microscope slides were subject to ZN staining. Fluorescent cultures were also tested for the presence of Map DNA by direct and nested PCR procedures for the detection of the *IS900* sequence, and by a direct PCR procedure for the detection of the *hspX* gene. Cultures that were positive for *IS900* and/or *hspX* by direct or nested PCR were tested for the presence of *hspX* messenger (m) RNA, by a reverse transcriptase (RT)-PCR procedure. Also, a 0.1 ml portion of each fluorescent culture was streaked on a slant of HEYA supplemented with mycobactin J at 2 mg/l and amphotericin B at 20 mg/l; and a 0.1 ml portion was inoculated into Middlebrook 7H9 broth supplemented with OADC enrichment, 0.2% (vol/vol) glycerol, and mycobactin J at 2 mg/l. Those slants and cultures were incubated at 37 °C for 20 weeks, with slants being examined weekly for presumptive Map colonies, and cultures being examined weekly for growth.

In addition, after 20 weeks of incubation, some MGIT cultures that did not fluoresce were tested for *IS900* by PCR and *hspX* by RT-PCR. Those cultures were all of the non-fluorescent cultures of raw or cooked outside round muscle, 15 cultures of raw or cooked, inside round, eye of round or ground muscle, and 12 cultures of control samples of raw or cooked muscle tissue or chopped lymph nodes. Samples from the latter two groups of samples, and cultures of not decontaminated or decontaminated portions from each sample were selected at random.

The PCR procedures for detection by direct and nested PCR of Map-specific *IS900* and *hspX* were as previously described (Gao et al., 2002; Ellingson et al., 2005). One-Step reactions were performed using the Qiagen One-Step RT-PCR Kit (Qiagen, Mississauga, Ontario, Canada). Reaction mixtures were prepared according to the manufacturer's instructions. Each mixture was prepared on ice and immediately transferred to a Geneamp PCR System 2400 thermocycler (Perkin Elmer, Boston, MA, USA). The one step RT-PCR to detect the *hspX* m-RNA used the forward and reverse primers 5′gaccggctatctgtggaac3′ and 5′ctcgtcggcttgcacctg3′, respectively; and 57.7 °C and 57.0 °C for the reverse transcription and annealing temperatures, respectively (Ellingson et al., 2005). One-Step RT-PCR products were resolved on agarose gels by electrophoresis.

3. Results

No aerobic bacteria were recovered from any sample of raw or cooked muscle tissue tested by the HGMF procedure at the times of sample collection.

When MGIT cultures were tested by PCR, all cultures that were positive for *IS900* or *IS900* and *hspX* were also positive for *hspX* by RT-PCR.

No Map colonies, or growth and/or PCR positive MGIT cultures were recovered from or obtained with samples of blood. Map colonies were recovered in high numbers from the faeces and livers of cows 1 and 2, and in low numbers from the kidneys of cows 1, 2 and 5 and the liver of cow 5 (Table 1). One or both MGIT cultures of liver from each of cows 3 and 4 were positive for growth, but only the cultures from cow 3 were also positive by PCR. When low numbers of Map colonies were recovered from a tissue, colonies were recovered more frequently from decontaminated than from not decontaminated portions of the sample.

No Map colonies were recovered from paired hamburgers spread with chopped mesenteric lymph nodes that were cooked to 70 or

Table 1
Numbers of *Mycobacterium avium* subsp. *paratuberculosis* (Map) colonies (cfu) recovered in duplicate tubes of Herrold's Egg Yolk Agar, from faeces or not decontaminated (ND) or decontaminated (D) portions of samples of organ tissues from five cows with advanced Johne's disease; and the portions which gave MGIT cultures that were A) positive for growth and by PCR, or were B) positive for growth only.

Faeces or tissue	Cows									
	1		2		3		4		5	
	ND	D	ND	D	ND	D	ND	D	ND	D
Faeces	•[a]	>200	•	>200	•	–[b]	•	•	–	–
Liver	>200	>200	>200	>200	A	A	B	–	4	6
Kidney	–	18	4	21	–	–	–	–	–	1

[a] Sample not available or not tested.
[b] Map not detected.

75 °C or from popliteal lymph nodes. Map colonies were recovered in high numbers from mesenteric and/or ileocaecal lymph nodes and/or raw hamburgers spread with chopped lymph nodes from cows 1, 2 and 4 (Table 2). Map colonies were recovered at lower or low numbers from the superficial inguinal and prescapular lymph nodes of cow 1 and the ileocaecal lymph nodes of cow 5. Map were detected in MGIT cultures of the mesenteric lymph nodes of cows 3 and 5; and in chopped lymph nodes from cows 3 and 5 in raw hamburger patties, or from cows 2 and 5 in patties cooked to 61 °C. Map colonies were not recovered from, and Map was not detected in MGIT cultures of hamburger patties spread with chopped lymph nodes that were cooked to 70 or 75 °C, or in cultures of popliteal lymph nodes.

Two Map colonies were recovered from the not decontaminated portion of a sample of raw outside round from cow 2. No Map colonies were recovered from any other muscle tissue homogenate. MGIT cultures that were positive for growth and for Map by PCR were obtained from not decontaminated portions of samples of raw outside round from cows 1 and 4, samples of outside round cooked to 61 or 70 °C from cow 4, a sample of raw inside round from cow 3 and a sample of raw ground meat from cow 2 frozen at −196 °C. MGIT cultures that were positive for growth and for Map by PCR and recovery of colonies on HEYA were obtained from not decontaminated portions of raw ground meat frozen at −18 °C from cow 1. A not decontaminated portion of a sample of each of frozen, raw outside round from cow 2 and frozen, raw eye of round from cow 3 gave MGIT cultures that were positive for growth but not by PCR.

Colonies were not recovered from any of the 46 control samples that were tested. The not decontaminate portions from 7 of these samples gave MGIT cultures that were positive for growth, but not for Map by PCR, recovery of colonies or ZN staining. No MGIT cultures of decontaminated portions were positive for growth.

MGIT cultures that were not positive for growth from 15 outside round, 13 other muscle or ground meat, and 12 control samples were tested by PCR. Of those MGIT cultures, 13 from outside round, 4 from other muscle and 4 from control samples were positive for Map by PCR. Seven of the outside round and one each of the other muscle and control samples that gave growth negative, PCR positive MGIT cultures had been cooked to ≥70 °C.

Table 2
Numbers of *Mycobacterium avium* subsp. *paratuberculosis* (Map) colonies (cfu) recovered in duplicate tubes of Herrold's Egg Yolk Agar (HEYA) from not decontaminated (ND) or decontaminated (D) portions of lymph nodes, or paired hamburger patties spread with chopped lymph nodes and not cooked or cooked to 61 °C; and the portions which gave MGIT cultures that were A*) positive for growth, and by PCR and recovery of colonies on HEYA, A) positive for growth and by PCR, or B) positive for growth only.

Lymph nodes	Cow									
	1		2		3		4		5	
	ND	D	ND	D	ND	D	ND	D	ND	D
Mesenteric	>200	>200	>200	>200	A	–[b]	–	–	A*	–
Ileocaecal	>200	>200	>200	>200	–	–	>200	>200	B	2
Sup. Inguinal	–	10	–	–	–	–	–	–	–	–
Prescapular	4	4	–	–	–	–	–	–	–	–
Chopped: raw	•[a]	•	>200	>200	3	52	>200	>200	A	A
Chopped: cooked to 61 °C	•	•	–	A	–	–	–	–	A	A

[a] Sample not available.
[b] Map not detected.

4. Discussion

As Map grows only very slowly samples of tissues or other materials are usually subjected to decontaminating treatments, to reduce the numbers of organisms that may overgrow any Map present in or on inoculated selective media that are incubated for several weeks. Decontaminating treatments will inactivate uncertain and possibly large fractions of Map populations present in samples (Dundee et al., 2001). Therefore, samples were collected using aseptic procedures so that portions of samples could be processed without as well as after decontamination, to obtain indication of the effects of decontamination of tissues on the recovery of any Map they may have harboured. The findings indicated that the decontamination procedure used in the study did not obviously affect the recovery of Map when the numbers in the tissues were high, as in mesenteric and ileocaecal lymph nodes. With organ and lymphatic tissues in which Map numbers were apparently low, Map were recovered from or detected only in decontaminated portions of samples in some instances. However, with such tissues in other instances and with all samples of muscle tissues Map were recovered from or detected in only not decontaminated portions of samples. Therefore, as could be expected, the findings suggest that recovery of small numbers of Map from tissues may generally be enhanced if tissue can be processed without a decontaminating treatment.

Map is an intracellular parasite that survives and proliferates within macrophage (Weiss and Souza, 2008). Map DNA was detected in preparations of white blood cells from about 11% of 262 Indian cattle of unspecified age or disease status (Bhide et al., 2006). Moreover, Map DNA in amounts corresponding to ≥75 Map cells/ml of blood was detected in blood from 34% of 361 patients with Crohn's disease, and 22% of 200 health blood donors (Bentley et al., 2008). It might then be expected that Map would be readily detected in blood from cattle with disseminated infections, and possibly in the blood of infected young and asymptomatic animals. That Map was not detected in blood from any of the cows with advanced Johne's disease may simply be a result of the few samples that were tested; but evidently Map cannot be detected reliably in the blood of even heavily infected animals by the methods used in this study.

Samples that gave MGIT cultures that were positive for growth but were negative for Map by PCR or for acid fast organisms must obviously be regarded as Map negative; but the status of cultures that gave MGIT cultures that were negative for growth and acid fast organisms but positive for Map by PCR is problematic. That samples of the latter type were more prevalent among samples from outside rounds than among samples from other muscles of Map infected animals is perhaps suggestive of very small numbers of Map being present in the samples that gave growth negative but PCR positive MGIT cultures, because Map was recovered or unambiguously detected in outside round more frequently than in other muscle tissues. The finding of control samples that gave growth negative but PCR positive MGIT cultures might then arise because the mesenteric lymph nodes of Map infected but non-shedding and asymptomatic young animals can be persistently colonized by Map (Ayele et al., 2004; Wu et al., 2007), and so Map DNA or possibly viable Map may be present at least occasionally within macrophage in other tissues of

young animals. However, it seems most unlikely that very small numbers in beef muscles tissue could survive cooking to ≥70 °C when Map in high numbers in lymphatic tissues were apparently all inactivated by cooking to such temperatures. Even so, the possibility of some Map surviving cooking of meat cannot be dismissed entirely, given that the survival of Map in pasteurized milk had been demonstrated (McDonald et al., 2005). The possible presence of small numbers of Map in meat tissues from young animals and the survival or otherwise of Map in cooked beef are evidently matters that require further investigation.

Cultivation of the fastidious and slow growing Map is uncertain, so consistent findings for the tissues from all five cows with advanced Johne's disease could not be expected. Nevertheless, the findings for samples from which Map were recovered or which gave MGIT cultures in which Map were detected unambiguously indicate that Map at numbers in excess of 10^3 cfu/g are likely to be found in only the liver and lymph nodes associated with the gut. Other tissues and lymph nodes would seem to be infected less frequently, with numbers at least an order of magnitude less than the numbers found in the heavily infected tissues.

Because frequent and heavy contamination of the mesenteric lymph nodes was to be expected, these as well as muscle tissues were used to investigate the effects of cooking. Medium rare or well done meat is obtained when steaks are cooked to temperatures at their centres of 63 or 71 °C, respectively (American Meat Science Association, 1995). Because the temperatures at the centres of steaks continue to increase after cooking is stopped (Gill et al., 2009), steaks were cooked to somewhat lower temperatures to ensure that any Map in the intact meat was not exposed to temperatures greatly above those recommended for medium rare or well done meat. Steaks were cooked to 75 °C to obtain meat that was very well done. To assure the microbiological safety of hamburger patties, it is recommended that they be well done. Therefore all patties, with or without the addition of chopped mesenteric lymph nodes, were cooked to a temperature somewhat above the recommended minimum temperature without holding, which is 71.1 °C, to allow for variability in the temperatures attained at different points within a hamburger patty (Passos and Kuaye, 2002). The findings indicate that while some Map may survive cooking of meat to a medium rare condition, their numbers would be greatly reduced; and that cooking to a well done condition could be expected to render meat free of viable Map.

Rapid freezing evidently had little or no effect on Map, as high numbers of Map were recovered from mesenteric lymph nodes frozen in liquid nitrogen before they were chopped and spread on hamburger patties as well as from lymph nodes that were not frozen. Any effect of slow freezing is uncertain, but it might be at most small, since Map was detected in ground beef frozen at −18 °C.

Although the findings of the study are limited, they indicate that consumers are unlikely to be exposed to large numbers of Map in intact or ground beef from Map infected animals, particularly if the meat is cooked to a well done condition. Livers and, possibly other organs from older animals that might have disseminated Map infections should perhaps be excluded from human consumption, as a precaution. The extent to which consumers may be exposed to small numbers of Map in beef requires further investigation, while whether or not exposure of consumers to small numbers of Map poses a health hazard has yet to be determined.

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