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Research Article

Apparent Prevalence of Beef Carcasses Contaminated with *Mycobacterium avium* subsp. *paratuberculosis* Sampled from Danish Slaughter Cattle

Hisako Okura,[1] Nils Toft,[1] Nicola Pozzato,[2] Annalucia Tondo,[2] and Søren Saxmose Nielsen[1]

[1] *Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark*
[2] *Istituto Zooprofilattico Sperimentale delle Venezie, SCT1, Via San Giacomo 5, 37135 Verona, Italy*

Correspondence should be addressed to Hisako Okura, hisako@life.ku.dk

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Presence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in beef has been reported as a public health concern because asymptomatically infected cattle may contain MAP in tissues that are used for human consumption. Associations between MAP carcasses contamination and animal characteristics such as age, breed, production type, and carcass classification were assessed. Cheek muscles from 501 carcasses were sampled cross-sectionally at a Danish abattoir and tested for presence of viable MAP and MAP DNA by bacterial culture and IS*900* realtime PCR, respectively. Cheek muscle tissues from carcasses of two dairy cows were positive by culture whereas 4% of the animals were estimated with ≥10 CFU/gram muscle based on realtime PCR. Age was found to be associated with carcass contamination with MAP. The observed viable MAP prevalence in beef carcasses was low. However, detection of MAP and MAP DNA in muscle tissues suggested that bacteremia occurred in slaughtered cattle.

1. Introduction

Paratuberculosis is a chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cattle and other ruminants. MAP infection in cattle is often given attention due to its possible connection to Crohn's disease (CD) in humans. CD is a chronic granulomatous ileocolitis, and patients with CD experience chronic weight loss, diarrhea, and chronic pain throughout their lives [1]. There is contradictory evidence that either support or dispute the association between MAP and CD [2, 3]. Human exposure to MAP from livestock could be via milk and meat products. For the latter, abattoirs are critical points to reduce MAP in meat and the risk of exposure to humans [4].

Infections in cattle occur primarily in calfhood, and may gradually progress to clinical disease after the incubation period, which ranges from a few months to the lifetime of an animal [5]. Although the exact time course is unclear, infected cattle may follow several stages corresponding to the changes that occur in histological and immune response such as establishment in intestines, cell-mediated immune responses, humoral immune reactions, and tissue destruction and bacteremia [6]. MAP in infected animals is primarily confined to the intestines and associated lymph nodes, but when the infection progresses, MAP is spread within the animal, although little is known about the timing and trigger mechanisms [7].

The infection prevalence of MAP at the slaughterhouse has been established, with previous studies reporting 16% and 1% in Danish dairy and nondairy cattle, respectively [8], 16% in culled dairy cattle in North America [9], and 34% in cull cows in the US [10]. However, the infection prevalence differs from carcass contamination prevalence, because not all infections have progressed to bacteremia.

The relationship between MAP infection and presence of MAP in different tissues of infected animals, in different stages of infection has been reported [11–15]. These studies show that MAP can be isolated from tissues other than

the primal infection site, including animals without clinical signs. For example, a study in the US reported disseminated infections in 57% of cows (12/21) with no clinical signs of disease [13]. Furthermore, MAP has been isolated from blood and diaphragm muscle of four slaughtered animals with only two animals exhibiting clinical signs of MAP infection [14].

Previous studies [10, 16], assessed the prevalence of MAP-contaminated carcasses were done without considering fecal contamination. Wells et al. [10] estimated that 80% of cull cows had MAP-positive hides whereas only 34% had MAP-positive ileocecal lymph nodes. This finding suggests that fecal contamination at the abattoirs is frequent. However, the prevalence of MAP contamination in tissues used for human consumption has not been established. It might be optimal if presence of MAP in carcasses can be linked with factors that are readily available at the farm or at abattoirs so that possible human exposure to MAP through beef could be minimized.

The objective of this study was to estimate the prevalence of MAP contamination in Danish beef carcasses and to characterize the contamination based on information collected at the farm or abattoirs, such as age, herd of origin, and fat code. Here, carcass contamination was defined as muscle tissue that tested positive by either culturing or PCR.

2. Materials and Methods

2.1. Collection of Samples. A cross-sectional sample from carcasses was obtained at a cattle abattoir in Denmark on two consecutive days in October 2009. All information was recorded with the animal identification number on the ear tag. On the slaughter line, samples were collected at the evisceration point, where postmortem examination was conducted. At the postmortem examination, an incision was made in the masseter muscle by a postmortem technician, and a piece of cheek muscle (approximately 20 grams each) from both sides were collected and placed in a separate container. Knives were washed in hot water (82°C) between animals. Swab samples were collected at the incision site of the cheek using a cotton swab (Transport Swabs, product number TSS, OXOID Ltd. Greve, Denmark) in order to examine if there was fecal contamination at the site. Data on birth date, herd of origin, breed, sex and fat code were obtained from the Danish Cattle Database. Fat codes indicate the amount of fat on the outside of the carcasses and in the thoracic cavity ranging from 1 (very thin) to 5 (very fat) [17].

2.2. Swab Preparation and Analyses. Swab samples were kept at 4°C until plating on MacConkey (for gram-negative bacteria) and Slanetz agar plate (for enterococci). Samples were incubated for 20 hours at 37°C followed by visual examination of the plates. Samples resulting in more than one colony on either agar plate were considered to have fecal contamination.

2.3. Muscle Samples Preparation and MAP Culture. Cheek muscle samples were stored at −18°C and transported to Istituto Zooprofilattico Sperimentale delle Venezie, Verona, Italy. Each sample was processed in parallel for MAP culture and realtime PCR, modifying the methods already validated for bovine fecal samples [18]. In brief, 3 grams of muscle were trimmed and added to 3 mL of sterile saline solution in a stomacher bag with a filter and homogenized for 2 min at maximum speed (BagMixer, Interscience, St. Nom, France). Then, 500 μL of the homogenate was placed into a 2 mL screw-cap tube for DNA extraction. The remaining sample was homogenized for a further 1 min with 8 mL of 0.75% hexadecylpyridinium chloride in half-strength brain heart infusion for decontamination. The liquid supernatant of the homogenate was transferred into 15 mL tubes, incubated at 37°C for 2 hours, and centrifuged at 900 g for 30 min. Pellets were resuspended in 600 μL of sterile water and 200 μL inoculated into each of 2 tubes containing 5 mL of 7H9+ liquid medium, specific for MAP culture. The 7H9+ liquid medium was prepared in house and contained 0.37% Middlebrook 7H9 powder (Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 8% OADC enrichment (Becton-Dickinson, Franklin Lakes, NJ, USA), 0.08% Casitone (Becton-Dickinson, Franklin Lakes, NJ, USA), 16% egg yolk, 0.4% Glycerol (Sigma-Aldrich, Germany), 1 μg/mL Mycobactin J (ID Vet, Montpellier, France), and 0.1 mL/tube PANTA PLUS (Becton-Dickinson, Franklin Lakes, NJ, USA). The tubes were incubated at 37°C and tested by realtime PCR at 4, 8, and 12 weeks of culture.

2.4. DNA Extraction and Realtime PCR Detection. DNA extraction was carried out starting from 500 μL of muscle homogenates or 300 μL of 7H9+ inoculated medium diluted with 200 μL of sterile water. Samples were added to screw-cap tubes containing 300 mg of glass beads (120–150 μm Sigma-Aldrich, Germany) and subjected to a bead beating step in the Fast Prep FP120 (Qbiogene, Irvine, CA, USA) instrument set twice at 6.5 m/s for 45 second. The DNA was then extracted with MagMAX 96 Viral Isolation Kit according to the manufacturer's instructions, using "MICROLAB STARLET" automated extraction platform (Hamilton Robotics, Bonaduz, Switzerland). For MAP detection, 900 nM primers (Map668F 5′-GGCTGATCGGACCCG-3′, Map791R-5′-TGGTAGCCAGTAAGCAGGATCA-3′) and 200 nM probe (Map718 5′-FAM-ATACTTTCGGCGCTGGAACGCGC-TAMRA) were used [18]. For the amplification of muscle samples, an internal control targeting endogenous bovine GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used by adding 10 nM of each primer (gapDHF 5′-GCATCGTGGAGGGACTTATGA-3′ and gapDHR 5′-GGGCCATCCACAGTCTTCTG-3′) and 50 nM of probe (5′-FAM-CACTGTCCACGCCATCACTGCCA-TAMRA). Amplification mix was completed with 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 2 μL of extracted DNA in a final volume of 10 μL. The program for realtime PCR was 2 min at 50°C followed by 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Realtime amplification was performed with Rotor-Gene 6000 (Corbett Life Science, Concorde, NSW, Australia).

2.5. Analytical Sensitivity of Realtime PCR and Quantification of MAP Cell Number in Artificially Contaminated Bovine Muscle Samples. Analytical sensitivity of the realtime PCR was evaluated using bovine meat from an uninfected cow spiked with tenfold dilutions of MAP ATCC 19698 (10^0 to 10^5 CFU), prepared according to Hugues et al. [19]. Colony counts of the inocula were performed in HEYM slants. Each sample was processed in triplicate, and results were calculated as CFU/mL of homogenate (ratio muscle/saline solution was 1 : 1). A standard curve for MAP numbers (CFU/mL) in beef samples was produced as a result of the threshold cycle (Ct) values.

2.6. Case Definitions. Eight definitions of MAP DNA occurrence in carcasses were explored regarding muscle PCR and swab test results. Ct values of 35, 36, 37, or 38 in the realtime PCR were considered to define a muscle tissue sample as positive. If one of 2 muscle tissue samples was positive in PCR, the carcass was defined as being contaminated with MAP DNA. Furthermore, the data were evaluated both including and excluding samples with fecal contamination. All eight definitions were explored for the assessment of possible risk factors whereas results of the assessment of the analytical sensitivity were used to estimate the MAP contamination prevalence.

2.7. Risk Factors. Four factors, namely, age, breed group, fat code, and herd type were examined in the statistical analyses. Age was dichotomised as 2 years old and older or less than 2 years old, because disease progression leading to bacteremia depends on time since infection. Sampled animals comprised 10 different breeds, and these were grouped into three: dairy (Danish Red, Holstein, and Jersey), beef (Angus, Charolais, Hereford, Highland, Limousine, and Simmental), and crossbred. Five levels of the fat code were grouped into three: 1 (very thin), 2 (thin), and 3 or higher (normal to fat), because infected animals with progressed disease would be expected to have reduced body fat. Three herd types (dairy, beef, and veal) were considered, because dairy herds have a higher MAP prevalence than beef herds [8], and veal herds are different from the other two in terms of age and management practices. "Dairy" was used for the animals born in and slaughtered from a milk-producing herd. "Beef" was for the animals born and slaughtered from a non-milk-producing herd. "Veal" was for the animals born in a dairy herd but moved to a nondairy herd.

2.8. Statistical Analyses. Prevalence of MAP DNA in tissue samples was calculated and further stratified by risk factors. Descriptive statistics were carried out by summarizing the PCR result in relation to the risk factors in combination with the different case definitions described above. A multivariable logistic regression including PCR result as response variable and the abovementioned four risk factors was carried out to determine factors affecting MAP DNA occurrence. All data analyses were performed using the free software R [20].

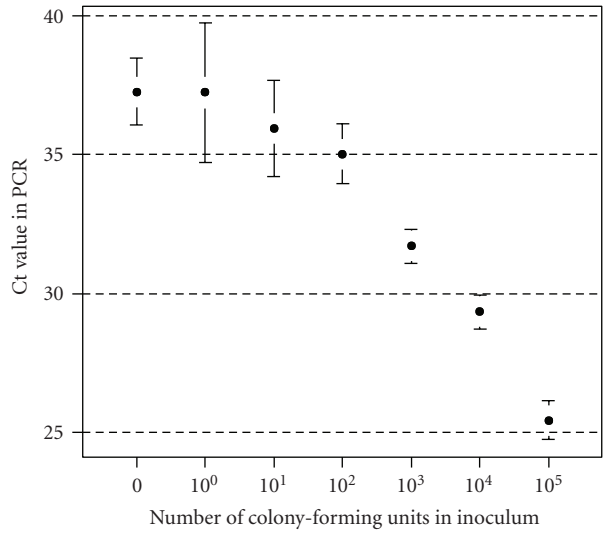

Figure 1: Quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cell number by IS*900* realtime PCR inoculating tenfold dilution of MAP ATCC 19698 artificially contaminated bovine muscle samples.

3. Results

In total, 1030 muscles tissue samples were collected from 515 animals. After excluding cattle for which identification number did not match information in the Danish Cattle Database, two samples from each of 501 beef carcasses were available. Of the 501 animals, 15 swab samples were not tested. For the 501 animals, ages ranged from 0.2 years to 14.3 years (median 3.2 years) with 304 older than 2 years of age. There were 362, 76, and 63 dairy, beef, and cross-bred breeds, respectively. Thirty-eight percent (192/501) of the animals were classified with fat code 3 or higher, and the median was 3 with first and third quartiles of 2 and 3, respectively. Three hundred and thirteen animals were from dairy herds, 73 were from veal herds, and 115 were from beef herds (Table 1).

Cheek muscles from two dairy cows (3.7 and 5.6 years of age) were positive by culture with no indication of fecal contamination. These samples were confirmed to be MAP using PCR, but the samples were not positive using the direct PCR. Association between culture positivity and risk factors (age, fat code, breed type, and herd type) was not observed due to small number of culture positives.

Concentration of MAP in beef samples was estimated as number of CFU per gram of beef from the standard curve obtained as a result of analyses of artificially contaminated bovine muscle samples (Figure 1). Exact quantification of MAP in beef at low concentrations ($<10^2$ CFU in inoculums) was impossible, but considering the Ct values in low concentrations, the detection limits would be around 10^1 CFU in inoculums of which corresponding Ct-values would be around 36. This cutoff was therefore selected for the prevalence estimation. From the standard curve, the concentrations of MAP in carcasses were very low (<10 CFU/gram; Figure 1).

Table 1: Number- and group-specific percentage (in brackets) of muscle tissue samples from slaughtered cattle from which *Mycobacterium avium* subsp. *paratuberculosis* DNA was detected by IS*900* PCR at threshold cycle value of 36, 37, and 38 by age, type, of herd, breed type and fat code. Positive results were defined in relation to possible fecal contamination to the tissue samples.

Variable	Level	Including samples with fecal contamination				Excluding samples with fecal contamination			
		Total no. in group	PCR threshold cycle value			Total no. in group	PCR threshold cycle value		
			36	37	38		36	37	38
		501	20 (4)	28 (6)	42 (8)	385	16 (4)	22 (6)	34 (9)
Age	<2 years	197	16 (8)	21 (11)	31 (16)	159	3 (1)	4 (2)	8 (4)
	≥2 years	304	4 (1)	7 (2)	11 (4)	226	13 (8)	18 (11)	26 (16)
Herd type	Dairy	313	17 (5)	22 (7)	30 (10)	235	13 (6)	17 (7)	23 (10)
	Beef	115	2 (2)	3 (3)	7 (6)	88	2 (2)	3 (3)	7 (8)
	Veal	73	1 (0)	3 (4)	5 (7)	62	1 (2)	2 (3)	4 (6)
Breed group	Dairy	362	18 (5)	26 (7)	35 (10)	278	14 (5)	20 (7)	27 (10)
	Beef	76	1 (1)	1 (1)	4 (5)	62	1 (2)	1 (2)	4 (6)
	Cross	63	1 (2)	1 (2)	3 (5)	45	1 (2)	1 (2)	3 (7)
Fat code	1	66	1 (2)	3 (5)	8 (12)	56	1 (2)	3 (5)	7 (13)
	2	162	9 (6)	14 (9)	15 (9)	125	7 (6)	10 (8)	11 (9)
	≥3	272	10 (4)	11 (4)	19 (7)	203	8 (4)	9 (4)	16 (8)

A total of 54 muscle tissue samples gave detectable fluorescence before a Ct-value of 40 was reached. The Ct-values of these samples were in the range from 31.9 to 39.9 with a median of 36.8. Distributions of Ct-values in the different categories are shown in Table 2. At 35 Ct-value, MAP DNA was detected in seven tissue samples from six dairy cows and one beef cattle with age between 1.0 and 6.3 years old, and these were positive without fecal contamination (1.4%). Overall, apparent prevalences of MAP-contaminated carcasses taking fecal contamination into account were 4%, 6% and 9% at Ct-values 36, 37, and 38, respectively (Table 1). At the selected cutoff of 36 Ct-values, 4% of the carcasses were deemed to be contaminated with ≥10 CFU/gram MAP muscle tissue.

Odds ratios and *P* values resulting from the univariable analyses suggested that age was consistently associated with PCR positivity regardless of choice of PCR cutoff as well as indication of fecal contamination: the odds of having MAP DNA were 2- to 10-times higher for cattle of 2 years old and older compared to young animals (Table 3). Therefore, in the multivariable analyses, it was decided to include age as a main effect in combination with other risk factors. The odds ratio suggested that breed was associated with PCR positivity at Ct-value of 37. However, production type and breed group were not included in the model at the same time because these two factors were highly correlated. The multivariable model of risk factors associated with the presence of MAP DNA in carcasses did not alter the univariable model with age as an explanatory variable (data not shown).

4. Discussion

The apparent prevalence of MAP culture positive was very low (2/501), and 4% (16/385) of carcasses were contaminated with ≥10 CFU/gram muscle among a cross-sectional sample of animals as deemed by PCR. The ideal cutoff for deeming an animal with MAP DNA contamination could not be determined, but the odds ratios (OR) from the univariable analyses (Table 3) suggested that it is likely between 36 and 38 Ct-values. The culture and the PCR indicated that concentrations in general were low.

A previous study describing isolation of MAP in muscle tissues reported that the prevalence of MAP in diaphragm muscle was 13% (6/47) among nonrandomly selected cattle at slaughter [14]. Other studies reported high prevalences of on-carcass contamination with MAP by testing swab samples before and after intervention and suggest that carcass contamination was a result of cross-contamination [10, 16]. Different prevalence of infection of cows could be an explanation, but also different sample site and way of sample collection could explain this difference because cheek muscle might be less likely to be exposed to contaminated environment than brisket or anal region.

True prevalences of MAP infection in adult Danish cattle have been estimated to be 16% among dairy cattle [8]. The observed apparent MAP prevalence of 4% of animals with ≥10 CFU/gram muscle in beef carcasses was lower than the infection prevalences of dairy cattle. However, this might reflect an actual low prevalence, because a recent study reported that MAP was isolated from muscle tissue and peripheral lymph nodes from 11% and 55%, respectively, of cattle with clinical paratuberculosis, which indicate a proportion of clinically diseased animals might have bacteremia [21]. The apparent prevalence was lows but the result indicates frequent occurrence of bacteremia in cattle to be slaughtered for human consumption.

Age was found to be associated with carcass contamination, which is not surprising because disease progression is

Table 2: Summary of threshold cycle value of IS*900* PCR to detect *Mycobacterium avium* subsp. *paratuberculosis* DNA in beef carcasses by age, herd type, breed group, and fat code of the cattle.

Variable	Level	PCR threshold cycle value					
		N	Mean	SD	Q_1	Median	Q_3
Overall		54	36.73	1.78	35.67	36.80	37.87
Age	<2 years	15	36.71	2.13	35.94	37.02	37.91
	≥2 years	39	36.73	1.65	35.75	36.56	37.86
Herd type	Dairy	36	36.36	1.80	35.47	36.23	37.54
	Veal	5	36.74	0.81	36.53	36.62	37.38
	Beef	13	37.74	1.64	37.20	37.88	38.93
Breed group	Dairy	42	36.48	1.78	35.57	36.30	37.66
	Beef	8	37.91	1.15	37.18	38.04	38.44
	Cross	4	36.90	2.13	36.27	37.20	37.83
Fat code	1	10	37.25	1.53	36.68	37.34	37.79
	2	19	36.06	2.03	35.02	36.28	37.00
	≥3	25	37.02	1.55	35.93	37.20	37.89

Table 3: Odds ratios (OR) and 95% confidence interval in parenthesis resulting from univariable logistic regression of risk factors for occurrence of *Mycobacterium avium* subsp. *paratuberculosis* DNA in beef carcasses.

Variable	Including samples with fecal contamination PCR threshold cycle value			Excluding samples with fecal contamination PCR threshold cycle value		
	36	37	38	36	37	38
Old						
<2 years	1	1	1	1**	1**	1**
≥2 years	2.7 (1.0–9.5)	2.0 (0.9–5.2)	1.9 (1.0–4.1)	10.1 (1.0–196.3)	3.4 (1.2–11.8)	2.5 (1.1–6.0)
Herd type						
Beef	1	1	1	1	1	1
Dairy	3.2 (0.9–20.7)	2.8 (1.0–12.1)	1.6 (0.7–4.2)	2.5 (0.7–16.3)	2.2 (0.7–9.6)	1.3 (0.5–3.3)
Veal	0.8 (0.0–8.3)	1.6 (0.3–8.9)	1.1 (0.3–3.7)	0.7 (0.0–7.5)	0.9 (0.1–5.9)	0.8 (0.0–2.8)
Breed group						
Beef	1	1	1	1	1**	1
Dairy	3.9 (0.8–71.1)	5.8 (1.2–104.4)	1.9 (0.7–6.6)	3.2 (0.6–59.2)	4.7 (1.00–85.7)	1.5 (0.6–5.1)
Cross	1.2 (0.1–31.0)	1.2 (0.1–31.0)	0.9 (0.2–4.2)	1.4 (0.1–35.7)	1.4 (0.1–35.7)	1.2 (0.1–31.0)
Fat code						
≥3	1	1	1	1	1	1
2	1.5 (0.6–3.9)	2.2 (1.0–5.2)	1.4 (0.7–2.7)	1.4 (0.5–4.1)	1.9 (0.7–4.5)	1.1 (0.5–2.5)
1	0.4 (0.0–2.2)	1.1 (0.3–3.7)	1.8 (0.7–4.3)	0.4 (0.0–2.5)	1.2 (0.3–4.2)	1.7 (0.6–4.2)

** Significant at $P \leq .05$.

known to be associated with age [7]. We also hypothesized that breed, production type, and fat code were associated with carcass contamination; therefore, we investigated the multivariable model including these factors. The odds ratios suggest that this may be true, but the association was not significant. Cattle testing positive for MAP DNA were generally 2 years and older, and these cattle were more likely to be from dairy herds and vice versa for beef cattle. Therefore, confounding between age and production type was present, and it could not be determined if age or production type was the actual explanatory variable. However, given that MAP infections are chronic, it appears more likely that age was the relevant predictor of MAP occurrence. No association between carcass contamination with MAP DNA and fat code of the carcasses was detected, which is consistent with findings of McKenna et al. [9].

One limitation of the study was that our muscle tissue sample was limited to cheek muscles. The two culture positive samples were confirmed to be MAP using PCR, but the samples were not positive using the direct PCR. A

reason for not being able to culture MAP from PCR-positive sample could be low concentrations or freezing treatment that is known to reduce MAP viability [22]. MAP load in two culture positive samples were also low. Previous studies that detected MAP in muscle from cattle with clinical signs also reported low concentrations [14, 21]. We collected muscle samples from both cheeks to increase sensitivity, because if bacteremia does occur, MAP should be distributed evenly in the body, but with respect to the difficulties in detection of MAP with low concentration, it might have been better with more samples or samples from other sites. The prevalence presented in the study was apparent prevalence. Test accuracy estimates (i.e., sensitivity and specificity) were not available. Therefore, the apparent prevalences could underestimate the true prevalences [21].

5. Conclusion

Two out of 501 (0.4%) animals tested positive for viable MAP, and 20/501 (4%) animals were found to have a concentration of ≥ 10 CFU/gram muscle tissue positive for MAP DNA, which indicates that the probability of carcass contamination with MAP and concentration of MAP is low. Age was the only factor that was found to be significantly associated with the occurrence of MAP DNA in muscle tissues; however, there were indications of other biologically important risk factors. The study provided quantitative data for presence of MAP in slaughtered cattle, but only age could be identified as an animal characteristic useful for risk characterization.

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