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Presence of *Mycobacterium avium* subs. *paratuberculosis* DNA in milk used to feed calves in Portugal

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This Technical Research communication describes results of a study aimed at detecting the presence of *Map* in milk fed to calves, and identifying possible risk factors for that presence. A questionnaire was performed on 37 dairy farms and waste milk samples were collected on 3 occasions separated by a minimum of 1 week. For farms not feeding waste milk, bulk tank milk samples were collected instead. A real time PCR for the detection of the *IS900* sequence was performed for the detection of *Map*. A majority of farms (89·2%) fed waste milk, with only one pasteurising the milk before feeding it to calves. Results of the PCR showed that 51·5% of the farms that were feeding waste milk had a positive result for *Map* on that milk. None of the studied risk factors were significantly associated with the presence of *Map* in milk samples, possibly due to the small number of farms entering the study. However, the prevalence of positive samples for *Map* on PCR was 3·5 times higher for farms that bought in animals from a single origin and 1·9 times higher for farms that bought from multiple farms, when compared with closed farms. Having a calving area for multiple cows also increased the risk of a positive *Map* result by 1·5 when compared with single pens. The risk of having a positive *Map* result on waste milk was 1·6 times higher for farms feeding that milk to male calves and 1·4 for farms feeding to both male and female calves, when compared with farms not feeding waste milk. This study highlights paratuberculosis as one of the potential risks of feeding waste milk to calves, and the need for mitigation strategies to be in place to avoid unnecessary disease transmission.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, Paratuberculosis, calf feeding, waste milk, milk direct detection.

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of a chronic granulomatous enteritis known as paratuberculosis, one of the most important diseases for cattle and the livestock industry worldwide, due to its considerable economic impact triggered by progressive and fatal weight loss of the animals, reduction of productivity, infertility and decreased milk production. The major routes of infection in ruminants are intrauterine and fecal-oral, through contact with fecal material from an infected animal and/or ingestion of contaminated colostrum and milk (Windsor & Whittington, 2009). Transmission of *Map* usually occurs during the first months of life, with calves under 6 months of age being the most susceptible to infection due to their immature immune systems, meaning an infective dose of about 50–10^3 CFU per calf (Windsor & Whittington, 2009).

Dairy farmers often feed calves with waste milk (Duse et al. 2015), a mixture of excess colostrum, transition milk and non-saleable milk from cows that have been or are still being treated with antibiotics. Feeding calves with waste milk may represent an economic benefit for farmers not only because it leads to savings in milk replacer, but also because feeding whole milk has been shown to lead to higher growth rates than feeding milk replacer with the

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Table 1. Results of the logistic regression based on questionnaire data and on the detection of Map in milk samples

						95% CI for Exp (B)	
Variable	Level	*N*	B	Sig.	Exp(B)	Lower	Upper
Buying in animals	No	19		0·453			
	Single origin	6	1·25	0·245	3·49	0·424	28·725
	Multiple origins	12	0·639	0·449	1·894	0·362	9·908
Type of calving area	Multiple cows	33	1·537	0·264	4·65	0·314	68·821
Waste milk fed to calves	No	4		0·491			
	Male calves	16	1·624	0·236	5·073	0·345	74·52
	All calves	17	1·434	0·288	4·594	0·276	76·381
Number of animals			−0·003	0·221	0·997	0·993	1·002

N, number of observations; B, coefficient; Sig, Significance; Exp(B), odds ratio; CI, confidence intervals.

same gross composition (Lee et al. 2009). This practice may, however, present a risk for calves due to the potential development of antimicrobial resistant faecal flora (Duse et al. 2015) and infection with pathogens, including *Map* that leads to the spread of paratuberculosis (Ridge et al. 2005).

The diagnosis of paratuberculosis is difficult and time consuming, due to the characteristics of the agent, and is influenced by the stage of the disease. Faecal culture is considered the gold standard method for *Map* diagnosis, but it may take up to 6 months for growth of the mycobacteria. The introduction of molecular diagnostic techniques has contributed to a faster and more sensitive detection of *Map* from biological samples including tissues, faeces and milk. Real time PCR is increasingly used for the direct detection of *Map* from biological samples due to its higher sensitivity and faster analysis time than conventional PCR and culture.

The present work aimed at evaluating the presence of *Map* in milk samples used to feed calves in selected farms by nested *IS900* real time PCR and culture, and to investigate potential risk factors for the presence of *Map* in those farms.

Materials and methods

Full details of the methodology are provided in Supplementary File Materials and Methods

Questionnaires. A questionnaire was performed on 37 Portuguese dairy farms in 3 geographic locations. It included questions related to possible risk factors or transmission of *Map* in dairy cattle comprising animal replacement policy, type of calving area, waste milk use and size of the lactating herd (Table 1). Other questions were included in the questionnaire to evaluate awareness of paratuberculosis by the farmers.

Milk samples. Waste milk fed to calves was sampled on three different days on each farm, separated at least 1 week between collections, to increase the likelihood that the source of the milk included different animals. Whenever waste milk was not fed to calves a single bulk tank milk sample was collected. Four milk samples had insufficient amount for processing and therefore 99 milk samples, comprising 95 waste milk samples and 4 bulk tank milk samples were analysed by culture and nested *IS900* real time PCR.

Preparation and culture of samples for Map isolation. Milk samples were prepared for culture according to Dimareli-Malli (2010), with minor modifications. The incubation was performed at 37 °C for up to 6 months.

Treatment of samples for genomic DNA extraction. All the milk samples were submitted to a first treatment procedure, as described by Gao & Colleagues (2007) with minor modifications. The DNA extraction was performed with the commercial extraction kit – Invisorb® Spin Tissue Mini Kit – protocol 1 (Stratec) with mechanical disruption and according to manufacturer's instructions with some modifications (Supplementary File). The genomic DNA was eluted with 100 µl of elution buffer and stored at −20 °C until being tested.

Amplification assays. A nested PCR approach was used, combining a first amplification step by standard PCR using the extracted DNA as template followed by a second amplification step by real time PCR where the amplified product was used as template. The amplification system was previously optimised for fecal samples (C. Leão, unpublished data) targeting the *IS900* multi-copy region. For the first conventional PCR step, external primers EXT-*IS900*-FW and EXT-*IS900*-RV were used, followed by real time PCR, using internal primers *IS900*QF/*IS900*QR and *IS900*QP targeted probe, described by Sidoti & Colleagues (2011). The limit of detection (LOD) of the assay for milk samples was determined with spiked milk samples (Supplementary file). *β-actin* gene-targeted probe/primers were used as internal control in the real time PCR to discount the presence of amplification inhibitors.

Statistical analysis

A multivariable logistic regression was performed using SPSS® version 22, to evaluate if the presence of potential

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risk factors for the occurrence of paratuberculosis at herd level, had a relation with the detection of *Map* through PCR in milk.

Results and discussion

The 37 farms included in the survey had between 16 and 715 lactating animals (median = 70) on the day the questionnaire was performed. Thirty-three out of the 37 respondents (89·2%) fed waste milk to calves, with 45·9% reporting to feed waste milk to both male and female calves and 43·2% reporting to feed waste milk only to male calves. Feeding waste milk to male calves alone is used by some farmers as an attempt to mitigate some of the possible problems of feeding waste milk, as male calves generally leave the farms at a young age for meat production.

All the farms that used waste milk to feed calves, included in it milk taken from animals during the treatment of clinical cases of mastitis or other diseases that were treated with antimicrobials, and milk taken during the withdrawal period of those treatments. Besides that, 66·7% of farmers also incorporated milk from cows with high cell counts to avoid penalties or maintain a bonus in milk price. Out of the 33 farms that fed waste milk to calves, only one pasteurised the milk before feeding it to calves, which was positive on the PCR after pasteurisation.

Twenty-four milk samples, representing 17 (51·5%) out of the 33 farms that fed waste milk were positive for nested *IS900* real time PCR. Only one of the four farms that did not feed waste milk to calves had a positive result on bulk tank milk for *Map*. None of the 99 cultured milk samples showed visible colonies after the incubation period of 6 months, with culture presenting a contamination rate of 18%. The difficulty in the isolation of *Map* from milk samples despite positive results being obtained through molecular detection is in accordance to Hanifian & Colleagues (2013), who reported ten times higher rates of positive results by real time PCR detection than with culture. Since these milk samples were collected from animals with other infections (e.g. mastitis) and from animals under treatment, the contamination rate of the cultures and the presence of antimicrobials could restrict the growth of a fastidious agent like *Map*. A positive culture result depends on the viability of the bacteria, the animal's infection load, the quality of the milk sample and the sample volume that is used in the analysis. It has been described that the number of cells that are excreted in milk of asymptomatic cattle is low, around 2–8 CFU per 50 ml of milk (Slana et al. 2008), and the volume that should be used for testing should thus be as high as possible, from 1 to 250 ml per sample (Slana et al. 2008). In this work we used only 20 ml of milk for culture and 10 ml of milk for DNA extraction, due to limited availability of higher sample volumes. Detection of *Map* DNA was thus used as a proxy for the presence of viable *Map*.

Feeding waste milk to calves has been found to be a significant risk factor for transmission of paratuberculosis (Ridge et al. 2005). Conflicting results have been published regarding the efficacy of pasteurisation to eliminate *Map* cells from milk, with some authors observing complete destruction of the agent either through pasteurisation at 65·5 °C for 30 min or through high temperature short time pasteurisation (Stabel et al. 2004), and other authors reporting incomplete destruction of the agent following heat treatment (Slana et al. 2008). Using waste milk to feed calves only from cows that tested negative for paratuberculosis is not a valid strategy as most of the diagnostic methods for paratuberculosis have fairly low sensitivities. Simultaneously, there is evidence that a high proportion of dairy cows that are negative for *Map* on fecal culture, have *Map* DNA on their colostrum, which suggests frequent environmental contamination of teats and milk with the agent (Pithua et al. 2011). In our study, the validity of milk pasteurisation as a preventative measure to control paratuberculosis could not be assessed as there were no positive cultures for *Map*, even though there were many positive milk samples when using the PCR. The risk of perpetuating paratuberculosis on these farms through the practice of feeding waste milk to calves is thought to be potentially high.

In terms of biosecurity, 48·6% ($n = 18$) of the farmers reported to have bought animals within the last 5 years. Out of the 18 farmers that kept an open farm, 66·7% ($n = 12$) bought animals from multiple sources, whereas the remainder bought animals from a single farm.

It was apparent from the results of the questionnaire that farmers' awareness on paratuberculosis was limited, with 78·4% ($n = 29$) of respondents stating they had no paratuberculosis on their herd, even though 32·4% ($n = 12$) stated they had seen, in the previous year, animals with a clinical picture highly suggestive of paratuberculosis (loss of body condition, chronic incurable diarrhoea and normal appetite). Only one farmer stated he did not know if there were infected animals in his herd. Many farmers also seemed to be unaware of the risks of feeding waste milk to calves, as 45·9% of all the farmers in the survey, fed waste milk to both male and female calves, with only one farm having the practice of pasteurising that milk.

None of the studied potential risk factors (Table 1) were significantly associated with the presence of *Map* in milk samples, therefore valid conclusions could not be drawn on potential risk factors for positive waste milk samples, probably due to the small scale of this study.

For the studied farms, the prevalence of having a sample positive for *Map* on PCR was 3·5 times higher for farms that bought in animals from a single origin and 1·9 times higher for farms that bought from multiple farms, when compared with closed farms. The fact that the risk seems higher from farms buying in from single sources than from multiple sources is contrary to what was expected. A previous survey of 122 dairy farms in Portugal showed that 45·9% of the herds had animals with positive serologies (Correia-Gomes et al. 2010). Given this prevalence, it is very likely that buying in replacements will ensure the introduction of

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animals with paratuberculosis into the herd. In our survey, there was an increased risk of positive *Map* samples for farms buying in replacements either from a single or from multiple sources.

Only four farms had single calving pens, limiting the usefulness of statistical analyses of single *v*. multiple animals per calving pen as a risk factor.

This was a small scale study aiming at investigating the risks of feeding waste milk to calves indiscriminately. The potential deleterious effects of this practice are not limited to the transmission of *Map*, with other infectious agents including *Mycoplasma bovis* and *Salmonella* spp., potentially being transmitted from adult cattle to calves in this way. Another possible risk of feeding waste milk to calves is the emergence of antimicrobial resistance in calves' faecal flora (Duse et al. 2015). Considered together, these risks should lead farmers to implement mitigation strategies. These might include pasteurisation of waste milk and colostrum at a temperature/duration that guarantee elimination of the agent, not feeding milk with antimicrobials and feeding milk replacer.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000164.

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References

Correia-Gomes C, Mendonça D & Niza-Ribeiro J 2010 Risk associations to milk ELISA result for paratuberculosis in dairy cows in northern Portugal using a multilevel regression model. *Revue de Médecine Vétérinaire* **161** 295–301

Dimareli-Malli Z 2010 Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected sheep and goats. *International Journal of Applied Research in Veterinary Medicine* **8** 44–50

Duse A, Waller KP, Emanuelson U, Unnerstad HE, Persson Y & Bengtsson B 2015 Risk factors for antimicrobial resistance in fecal *Escherichia coli* from preweaned dairy calves. *Journal of Dairy Science* **98** 500–516

Gao A, Mutharia L, Raymond M & Odumeru J 2007 Improved template DNA preparation procedure for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by PCR. *Journal of Microbiological Methods* **69** 417–420

Hanifian SS, Khani S, Barzegari A & Shayegh J 2013 Quantitative real-time PCR and culture examination of *Mycobacterium avium* subsp. *paratuberculosis* at farm level. *Veterinary Microbiology* **162** 160–165

Lee HJ, Khan MA, Lee WS, Yang SH, Kim SB, Ki KS, Kim HS, Ha JK & Choi YJ 2009 Influence of equalizing the gross composition of milk replacer to that of whole milk on the performance of Holstein calves. *Journal of Animal Science* **87** 1129–1137

Pithua P, Wells SJ, Godden SM & Stabel JR 2011 Evaluation of the association between fecal excretion of *Mycobacterium avium* subsp *paratuberculosis* and detection in colostrum and on teat skin surfaces of dairy cows. *Journal of the American Veterinary Medical Association* **238** 94–100

Ridge SE, Baker IM & Hannah M 2005 Effect of compliance with recommended calf-rearing practices on control of bovine Johne's disease. *Australian Veterinary Journal* **83** 85–90

Sidoti F, Banche G, Astegiano S, Allizond V, Cuffini AM & Bergallo M 2011 Validation and standardization of *IS*900 and F57 real-time quantitative PCR assays for the specific detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis*. *Canadian Journal of Microbiology* **57** 347–354

Slana I, Paolicchi F, Janstova B, Navratilova P & Pavlik I 2008 Detection methods for *Mycobacterium avium* subsp. *paratuberculosis* in milk and milk products: a review. *Veterinarni Medicina* **53** 283–306

Stabel JR, Hurd S, Calvente L & Rosenbuch RF 2004 Destruction of *Mycobacterium paratuberculosis*, *Salmonella* spp., and *Mycoplasma* spp. in raw milk by a commercial on-farm high-temperature, short-time pasteurizer. *Journal of Dairy Science* **87** 2177–2183

Windsor PA & Whittington RJ 2009 Evidence for age susceptibility of cattle to Johne's disease. *Veterinary Journal* **184** 37–44

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