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Article

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* and associated risk factors in dairies under mechanical milking parlor-systems in Antioquia, Colombia

Nathalia M. Correa-Valencia [a]

Nicolás F. Ramírez-Vásquez [a]

Jorge A. Fernández-Silva [a*]

[a] Universidad de Antioquia. Facultad de Ciencias Agrarias. Centauro, Escuela de Medicina Veterinaria, Carrera 75 # 65 - 87, Ciudadela Robledo, Medellín, Colombia.

*Corresponding author: jorge.fernandez@udea.edu.co

Abstract:

The present study aimed to determine *Mycobacterium avium* subsp. paratuberculosis (MAP) prevalence according to environmental samples and to explore the herd-level risk factors associated to MAP infection in dairy herds under mechanical milking parlor and pasture grazing-based systems. The study herds (n= 94) were located in 60 districts from five municipalities in the Northern region of the province of Antioquia, Colombia. Herds were visited once in 2016 to collect two composite environmental samples and to complete a risk assessment questionnaire. MAP identification was carried out using a quantitative real-time PCR method based on the IS*900* sequence. A herd was considered as MAP-positive if one or both of the environmental samples were found positive by the molecular technique. The information on risk factors was analyzed using a multivariable logistic regression model. The apparent herd-level prevalence found was 14.9 % (14/94; 95 % CI: 7.7-22.1), ranging from 0 to 33.3 % at municipality-level. Herds where other than Pure-Holstein breeds were predominant (i.e. Jersey, Jersey crossbreeds) were more likely to be MAP-qPCR positively infected than those on which where pure-Holstein cattle was predominant (OR=3.7; 95 % CI: 1.1-15.2). The present study reports MAP prevalence in dairy herds in the province of Antioquia (Colombia), and the association between MAP environmental positivity with the predominant breed in the herd.

Key words: Environmental sampling, Holstein, Jersey, Johne's disease.

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Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causal agent of Johne´s disease (JD)[1], a slow-developing chronic granulomatous enterocolitis[2]. MAP is resistant to both environmental and chemical changes and can survive in the environment for up to a year[3,4]. The disease has a worldwide distribution and related production losses are also described[5,6].

Each of the currently available MAP-diagnostic tests presents advantages and disadvantages, depending on the matrix and the different stages of the infection and subsequent illness[7,8]. Molecular detection of MAP using polymerase chain reaction (PCR) on environmental samples has been proposed for herd screening[8,9], with equivalent results to those obtained by culture[10]. The sensitivity (Se) of the PCR (in every format) can vary due to the irregular fecal shedding of the organisms, whereas its specificity (Sp) is close to 100 % in all stages of the disease[11,12]. Quantitative real-time-PCR (qPCR) method has been found to be sensitive (≈60 %) and specific (≈97 %)[13,14], also allowing MAP detection and quantification on complex matrixes (e.g. milk, fecal samples)[15-17]. The analysis of environmental samples using qPCR is considered nowadays as a cost-saving and easy-to-use approach to diagnose JD at herd-level and to classify the herd as infected or not, since it does not require sample collection from individual animals, reducing the inherent stress of the sampling process[16,18].

The JD herd-level prevalence worldwide seems to be >18 %, with reports >50 %[19-21]. In Colombia, an apparent herd-level seroprevalence appeared to be >50 %, according to ELISA-based studies[22,23]. Nevertheless, other studies have reported lower prevalences, such as 3.6[24] and 4.1 %[25]. It seems that the range of possible results about prevalence estimation in the country is wide, and depends largely on the diagnostic test used and on the population of study. In Colombia, data on animal or herd-level risk factors on JD in dairy herds are still limited and dairy production systems are diverse. Hence herd-level management practices and risk factors, which differ between herds and dairy production systems[26,27], demands the local definition of risk factors for the disease considering the local diversity of dairy production systems. This diversity is often overseen and reports on prevalence and risk factors commonly ignores this fact, delivering data which cannot be compared with other studies and results, even in the same region or country. In a previous recent cross-sectional study, 292 dairy herds with on-paddock milking facilities —mobile units, located in 61 different districts from six municipalities in Northern Antioquia (Colombia) were sampled with one composite environmental sample

containing material from at least six different sites (subsamples) of the concentration of adult cattle and/or high traffic areas in grazing paddocks. In this study, herds with a history of mixed farming of cattle with other ruminants had higher odds of being MAP infected than herds without this feature[25].

Other very common dairy production system in Northern Antioquia and in whole Colombia, based its milk production on mechanical milking parlor and pasture grazing-based systems. This system is hypothesized to have different prevalence levels as well as different herd-level risk factors in comparison to dairy herds with on-paddock milking facilities —mobile units, which was previously studied[25]. Therefore, this cross-sectional study aimed to determine MAP herd-level prevalence according to IS*900*-qPCR results on environmental samples, and to explore herd-level risk factors associated to MAP infection in dairy herds under mechanical milking parlor-systems of the Northern region of the Province of Antioquia (Colombia).

Material and methods

Study design and herd selection

A cross-sectional probabilistic study design was carried out in the Northern dairy region of the Province of Antioquia (Colombia) in 2016. Selected herds were distributed into five municipalities (San Pedro de los Milagros, Entrerríos, Santa Rosa de Osos, Donmatías, and Belmira), known for their considerable volumes of dairy production. The study area is found between 1,090 and 2,979 m asl, and the environmental temperature ranges from 12 to 16 °C. According to the Caldas-Lang climate classification, Santa Rosa de Osos, San Pedro de Los Milagros, Entrerríos, and Donmatías municipalities are classified as cold-humid, and Belmira municipality as cold-very humid[26].

The herd was considered as the unit of analysis. The study was performed under a stratified random sampling design, with restitution and without replacement. A municipality- and district-level proportional distribution was considered in the study design, according to the adult cattle population in each level (cows and bulls >2 yr of age)[27]. The selection of districts to be sampled into each of the five municipalities was defined according to their specific weight into the corresponding municipality, considering the first districts with the largest adult bovine population, until the quantity of their census reached the 70 % of the population into each municipality.

Sample size was defined according to a formula for prevalence estimation from a finite population[28], including *a priori* JD-prevalence proportion estimation of 0.118 (11.8 %) from a previous report in the study region[25] —achieved under similar methodological

conditions and population, a 95% confidence level, and a maximum acceptable error rate of 7 %. The upper limit of such report was considered. The sampling frame referred to 7,794 herds registered on the foot-and-mouth disease vaccination records of the five municipalities of study[27].

From the listed herds, 94 herds in 60 districts were randomly selected, according to the sampling strategy and inclusion criteria (i.e. having adult cattle, mechanical milking in parlor facilities and pasture grazing-based systems, geographic accessibility, no previous history or report of JD or MAP detection by any method, disposition of the owner to participate).

Collection of the sample and questionnaire

Environmental sampling was carried out as reported previously by the literature[18,29], with some modifications due to particularities in the productive systems and facilities in the study region (e.g. maternity, quarantine and/or nursing area not always defined) and to budget restraints.

Each study herd was visited once to collect two composite environmental samples and to fulfill the questionnaire. The first composite sample contained subsamples from at least six different points of concentration of adult cattle/high traffic areas (e.g. paddock, areas nearby waterers and feeders, alleyways, gutters, milking parlor holding areas). Each subsample was collected considering those not being previously exposed to direct sunlight. The second composite sample contained manure from the milking parlor collected from the manure storage lagoon, after mixing its content for at least 5 min before sampling. The subsamples from the lagoon were obtained from six different places of the perimeter by submerging the sampling container up to 10 cm beneath the surface. Each environmental sample was collected using a clean disposable plastic glove. Subsamples of each of the two collection places were pooled and mixed by hand at the farm. Then, approximately 20 g of each of the two pool samples (separately) was placed into a container. Definitive samples were preserved in refrigeration at 4 °C during transport to the research laboratory, where they were homogenized by hand for 5 min and then stored at -20 °C until DNA extraction.

The same one-page questionnaire used and reported previously[25] was applied herein (available upon request).

Laboratory analysis

Laboratory analysis was carried out as previously described[25]. Briefly, a commercial DNA preparation kit (ZR Fecal DNA Kit™, Zymo Research, CA, USA) was used for the DNA isolation, and the protocol included a bead-beating *prior* step (Disruptor Genie® 120V, Thomas Scientific, Swedesboro, NJ, USA). A NanoDrop 2000® spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the purity and yield of nucleic acids at two wavelengths (A260 and A280 nm). DNA integrity was confirmed using an only-agarose gel on a representative sub-sample of each extraction batch (10 %). DNA extraction efficiency was confirmed by PCR using bacterial constitutive genes to the same sub-samples mentioned above. The extracted DNA was preserved at -20 °C until IS*900*-qPCR analysis (Bactotype MAP PCR Kit®, Qiagen, Leipzig, Germany). The analyzed sample was considered as *positive* when a FAM/MAX channels signal was produced or *strongly positive* if a FAM-only signal was emitted, with a Ct≤40 and a sigmoid-pattern curve, according to MIQE guidelines[30].

Statistical analysis

Statistical analysis was carried out as previously described[25]. The independent variable was the IS*900*-qPCR MAP-infection herd-status (positive/negative). All the information was analyzed using Stata 15.0 (StataCorp, 2017, College Station, Texas, USA) for the descriptive and regression modeling. Descriptive statistics were computed for all the variables of interest. A complex survey analysis was considered, according to a district-level cluster-effect and to the stratified design of the study. Univariable analysis was performed to assess unconditional associations between the outcome (MAP-herd status) and each independent predictor using simple logistic regression. Associations with a $P \leq 0.20$ were considered for inclusion in the multivariable logistic regression model. Evaluation of potential confounders was then performed by assessing the change in the β-coefficient of the variables of the adjusted model compared to the non-adjusted model. The variables to be explored as confounders (i.e. herd size, predominant breed) were considered according to literature. Biologically plausible interactions were studied between significant variables from the multivariable models, as well as the 2-way interactions between significant predictors with a significant unconditional association with the dependent variable. Confounders were only retained if a change greater than 15% was observed, regardless of the significance of the coefficient of the confounding variable in the model. Independent variables included in the final model were selected according to a backward-stepwise procedure (entry $P=0.20$; removal $P=0.25$). The final model is presented considering Odds Ratios (OR) with 95 % CIs. The model fit was assessed using the Hosmer-Lemeshow goodness-of-fit test[28].

Results

Two environmental samples were collected from each of 94 dairy herds under mechanical milking parlor and pasture grazing-based systems, located in 60 different districts in five municipalities of the Province of Antioquia (Colombia). None of the herds were housed or semi-housed. The 2.1 % (2/94) of the primarily eligible herds did not approve to be visited when they were first contacted by phone and, a 6 % of the phone numbers were out of service/not registered. The non-participating herds were considered as *big* dairy herds (>30 milking cows) for the Colombian context, and mainly located in the municipalities of Donmatías and Entrerríos, according to the Province´s census records. The apparent herd-level prevalence found was 14.9 % (14/94; 95 %CI: 7.7-22.1), ranging from 0 to 33.3 % at municipality-level (Table 1). Tables 2 and 3, show the herd-level characteristics and management practices considered as predictors for the MAP-risk factor approach.

Table 1: Municipality-level prevalence of *Mycobacterium avium* subsp. paratuberculosis in the Province of Antioquia, Colombia (2016)

Municipality	**Sample weight (%)**	**Herds of study**	**No. of positive herds (%)**
Belmira	3.2	3	0 (-)
Santa Rosa de Osos	16.0	15	0 (-)
Entrerríos	26.6	25	6 (24.0)
San Pedro de Los Milagros	41.5	39	4 (10.3)
Donmatías	12.7	12	4 (33.3)
Total	100	94	14 (14.9)

Table 2: Herd-level characteristics in dairies from the Northern region in the Province of Antioquia, Colombia (2016)

Predictor	**CAT**	**PH (n)**	**NH (n)**	**N**	**DIS (%)**	**OR (95%CI)**	***P*-value**
Herd size	≤ 30	2	15	17	18.1	1.4	0.514
	>30	12	65	77	81.9	(0.3-6.9)	
Predominant breed	Pure	10	71	81	86.2	3.2	0.096*
	Holstein	4	9	13	13.8	(0.8-12.2)	
	Other[a]						
Availability of veterinary assistance	Yes	12	73	85	90.4	0.6	0.871
	No	2	7	9	9.6	(0.1-3.1)	

Cattle purchasing practices (use of replacement calves/heifers in the last 10 yr)	Yes No	4 10	37 43	41 53	43.6 56.4	0.5 (0.1-1.6)	0.226
Foreign animals grazing in own pastures	Yes No	1 13	1 79	2 92	2.1 97.9	6.1 (0.4-103.3)	0.262
Own animals grazing in non-proper pastures	Yes No	0 14	4 76	4 90	4.3 95.7	0	IN
Co-farming in the last 2 yr of the cattle with other MAP-susceptible ruminants (e.g. goats, sheep, buffaloes)	Yes No	3 11	17 63	20 74	21.3 87.7	1.0 (0.3-4.0)	0.938
Ruminants species co-farming with the cattle in the last 2 yr	Goats Sheep Sheep and goats Not applicable	2 0 1 11	8 8 1 63	10 8 2 74	10.6 8.5 2.2 78.7	5.7 (1.5-20.9) 0 1.4 (0.2-11.6)	0.099 IN 0.773
Good farming practices-status (GFP; according to the ICA)	Yes No	9 5	33 47	42 52	44.7 55.3	2.6 (0.8-8.4)	0.111*
Bovine tuberculosis status (tuberculosis-free according to the ICA)	Yes No	11 3	57 23	68 26	72.3 27.7	1.5 (0.4-5.8)	0.572
Producer´s knowledge about the disease	Some[b] Never heard about it before	4 10	0 70	14 80	14.9 85.1	0.4 (0.1-1.4)	IN
JD-compatible symptoms´ history	Yes[c] Never	3 11	17 63	20 74	21.3 87.7	1.0 (0.3-4.0)	0.938

CAT= categories; PH= positive herds; NH= negative herds; DIS= distribution; IN= inestimable. ICA= Instituto Colombiano Agropecuario. OR= Odds Ratio. CI= Confidence interval.

[a] Includes: Pure Jersey and Jersey- crossbreeds. [b] Includes: Recognizes the name only, some basics, and fairly knowledgeable. [c] Includes: At present and/or in the last 2 yr. * Variables used for the multivariable analysis ($P \leq 0.20$).

Table 3: Herd-level management practices in dairies from the Northern region in the Province of Antioquia, Colombia (2016)

Predictor	CAT	PH (n)	NH (n)	N	DIS (%)	OR (95% CI)	*P*-value
Manure spreading as fertilizer in the pastures	Yes	14	77	91	96.8	0	IN
	No	0	3	3	3.2		
Typical time of separation of the newborn calf from their dam after birth (in days)	≤ 1	4	17	21	22.3	0.68 (0.2-2.4)	0.544
	≥ 2	10	63	73	77.4		
Calves ≤ 6 mo old in direct contact with adult cattle	Yes	2	10	12	12.8	1.2 (0.2-6.0)	0.854
	No	12	70	82	87.2		
Source of colostrum fed to calves	From multiple cows	0	0	0	-	0	IN
	From its own dam	14	80	94	100.0		
Source of milk fed to unweaned calves	Unsalable milk	5	30	35	37.2	1.08 (0.3-3.5)	0.899
	Other sources[a]	9	50	59	67.8		

CAT= categories; PH= positive herds; NH= negative herds; OR= Odds Ratio. CI= confidence interval; IN= inestimable.

[a] Includes: Milk without antibiotic (salable milk) and milk replacer. * Variables used for the multivariable analysis ($P \leq 0.20$).

The variables with an average of "0" between herds with MAP-positive/negative status were excluded from logistic regressions (i.e. own animals grazing in non-proper pastures, producer´s knowledge about the disease, manure spreading as fertilizer in the pastures, source of colostrum fed to calves). The variables herd size and cattle purchasing practices were included in the analysis as potential confounders, but the relative change in the coefficients was <15%, so they were not furtherly explored.

The final multivariable logistic regression model for MAP-positive status in the dairies of study showed that herds where other than pure-Holstein breeds were predominant (i.e. Jersey, Jersey crossbreeds) were more likely to be MAP-qPCR positively infected using environmental sampling than those on which where pure-Holstein cattle was predominant (OR= 3.7; 95 % CI: 1.1-15.2).

Discussion

The present study was carried out to determine MAP herd-level prevalence according to environmental samples in 94 herds in five different municipalities. In addition, the study aimed to explore the herd-level risk factors associated with MAP infection in dairies under mechanical milking parlor and pasture grazing-based systems in the Northern Antioquia, Colombia. All herds found positive to MAP-qPCR were considered as infected, based on the fact that a MAP-elimination source leads to environmental fecal contamination, and therefore to the risk of ingestion by susceptible cattle[31].

The apparent MAP herd-level prevalence of 14.9 % estimated from the present study (0-33.3 % at municipality-level), appears to be lower than those reported for cattle in North American, European, and Latin American and Caribbean cattle regardless of the dairy or beef production system[19-21]. At a national scale, results from a recent study carried out in the same region in dairy herds with in-paddock milking facilities found an apparent prevalence of 4.1 %[25] based on molecular detection of MAP in environmental samples using a quantitative real-time PCR method based on the IS*900* MAP-sequence.

Differences in prevalence estimations of these two dissimilar dairy production systems (even being both under rotational grazing systems in most cases) and milking procedures could be due, hypothetically, to a higher metabolic load and consequent stress for individuals, which must walk at least twice a day to and from the milking parlor compared to those cows in dairy herds with in-paddock milking facilities, in which cows remain on pastures grazing most of the day and it is the milker who approaches them for milking. Cows that have to walk several times per day could have a compromised immunity that could favor the success of intestinal colonization by MAP and the formation of granulomatous lesions, and the consequent elimination of the agent to the environment[2,32], as well as the infection by other pathogens. In addition, the higher apparent prevalence could be due to a higher probability of detection of positive herds in the environment when two samples of each are collected, as followed in this case[16,33]. These proposed arguments need further research approaches.

A precise place known as appropriate to define a herd-level MAP-positive finding is the manure storage lagoon[34,35]. Its considerations in the study region seem not to be a representative characteristic of the local/regional dairy systems in Colombia, taking into account that only 1 out of 4 dairies in the Province counts on this facility[27]. Nevertheless, such dairies were a representative source of the positive findings, since 8 of the 14 herds found as MAP-qPCR positive were detected using the samples from the lagoons, whereas five of the positives were from the adult cattle concentration and/or high traffic area, and one from both sampling places. This may be an additional explanation when comparing the prevalence result of this work with that previously reported[25], as mentioned.

It was found that herds where other than pure-Holstein breeds were predominant (namely, Jersey and Jersey crossbreeds) were more likely to be MAP-qPCR positively infected compared to those on which pure Holstein was the predominant one. An apparent higher susceptibility to JD for other than pure Holstein-breed cows have been reported in previous studies[36-38]. Channel Island breeds (i.e. Jersey, Guernsey) have been suggested to be more susceptible to JD, based on evidence that the clinical disease has more frequently been reported in these breeds than any other breeds[37]. However, the reason for this remains unknown. It has been hypothesized that this susceptibility may be related to increased exposure rather than increased susceptibility or may be confounded by some factors that play important role in the development of clinical disease such as lower culling rate in Channel Island breeds, just to give an example, but that information is not available from this study. Susceptibility to infection is suspected, then, to have a genetic component, and moderated values for heritability of infection and susceptibility have been reported and some approaches have been made so far on the topic.

In this respect, the use of genetic selection as a control tool for JD is a relatively new approach. The phenotype (infection status) shown by some animals is a combination of genetically determined factors (susceptibility/resistance/tolerance genes) and environmental factors (exposure to MAP)[12].

Susceptibility is evidenced by infection and progression to the clinical stage; resistance is characterized by the absence of infection or successfully fighting an infection and eliminating the pathogen from the body; and, tolerance is characterized by infection and a subclinical status. It is expected that the genetic variations of the host contribute to modify the response of the animal to the exposure to the agent and reaching one of the three triggering challenge scenarios[39]. In the case of JD, the research objectives so far have been focused mainly on the assessment of susceptibility. This is given mainly for reasons of practicality, since resistance or tolerance to infection should be evaluated through challenge studies, in which the animals are exposed to equal doses of the pathogen and subsequently their response would be evaluated over a long period of time, being an expensive approach (due to the type of species), in the long term (due to the pathogenesis of the disease), and to reach acceptable conclusions the group of animals must be large[40]. Nevertheless, stablishing a more MAP-resistant population through breeding programs should not be considered a complete solution to control the disease, but rather as a tool to prevent or reduce the incidence of infection[41].

Milk and colostrum can carry MAP, because of fecal contamination of teats or by being excreted from the udder[7]. From a local point of view, other authors have reported that the odds of being a seropositive herd were lower in those feeding calves with pooled colostrum from several cows compared to those to herds feeding calves with colostrum from their own dams[42]. This previous study was carried out on 14 dairies, located in the municipalities of Belmira and San Pedro de los Milagros (Province of Antioquia), two of the five municipalities included in this study. Their results are in contrast to previous knowledge of the risk of being seropositive represented by the use of colostrum from

multiple cows *vs* own dam´s[7]. Results in this work reported that all the colostrum given to the calves is from their own dams (100 % of the herds). Other studies have related feeding antibiotic-contaminated or other discard-milk to young animals to be a significant risk factor for MAP spread[43]. The results indicated that milk replacers and salable milk (without antibiotics) were the main sources used to feed unweaned calves. Nevertheless, according to the experience, to use discarded milk to feed the calves is still a common practice in the systems of study in Colombia, increasing the odds of within-herd transmission of, not only MAP, but other infectious agents.

Conclusions and implications

The apparent prevalence found in the herds with in-paddock milking facilities of the present study was 14.9 %, varying from 0 to 33.3 % between the municipalities of study. In addition, it was found that dairies -where other than pure-Holstein breeds were predominant- were more likely to be MAP-qPCR positively infected using environmental sampling than those on which pure-Holstein was the predominant one (OR= 3.7; 95 %CI: 1.1-15.2), which does not mean that Holstein cattle are resistant to MAP infection. Nevertheless, this feature should be taken into account for JD´s control, particularly in dairies in Colombia under the same dairy production system than the ones considered herein.

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Conflict of interest

The authors state that they have no conflicts of interest to declare.

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