Original Article

Prevalence of Paratuberculosis in Dairy Cattle in Ecuador

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Abstract

Background: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causal agent of paratuberculosis, a chronic infectious contagious disease of the intestinal tract of ruminants that are also associated with Crohn's disease in humans. The existence of paratuberculosis in Ecuador is virtually unknown; hence, the present study was performed to gain insight into the prevalence of this disease. **Methods:** Three dairy cattle farms in different geographic regions in Ecuador were investigated for the infection with MAP, and 600 blood samples, 200 of each cattle herd, were processed with an indirect enzyme-linked immunosorbent assay. Fecal samples of the seropositive cows were processed for culture on modified Löwenstein–Jensen medium. **Results:** One hundred and fifty bovines (25%) resulted seropositive and we confirmed with culture the presence of MAP in 4.7% (7/150) of the seropositive cows. Approximately 20% of the fecal samples of seropositive cows yielded nontuberculous mycobacteria (NTM) species including *M. avium* subsp. *avium,* a NTM species closely related to MAP. **Conclusions:** The seroprevalence of paratuberculosis in this first study for Ecuador is high (25%). We discuss a possible interference of NTM species, isolated from fecal samples, with the diagnosis of paratuberculosis. With this report, a baseline study, we confirm for the first time the presence of paratuberculosis in Ecuador, and we provide the necessary information for future studies and control of this disease.

Keywords: Ecuador, indirect enzyme-linked immunosorbent assay, *Mycobacterium avium subsp. paratuberculosis*, nontuberculous mycobacteria, paratuberculosis or John's disease, polymerase chain reaction-insertion sequence 900

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Introduction

Paratuberculosis or Johne's disease is a chronic infectious disease that primarily affects the small intestine tract of ruminants, produced by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), responsible for considerable economic losses for the livestock (decreased milk production and slaughter value).[1] MAP affects domestic and wild ruminants, mainly sheep, goats, and cattle. The main symptomatology is the development of a granulomatous enteritis, weakening, diarrhea, and death due to cachexia.[2-4] It also has been suggested that paratuberculosis is a zoonotic disease, and there may be an association between Crohn's disease (CD) and Johne's disease,[5] and MAP has been cultured from the intestinal tissues and the blood of CD patients.[6]

MAP is a Gram-positive obligate intracellular pathogen that needs mycobactin (a siderophore used to shuttle free extracellular iron ions into the cytoplasm of mycobacterial cells) for "*in vitro*" growth, and therefore, is incapable to grow outside of its host.[7] Fecal cultures and specific antibody detection have been the most common diagnostic methods used for the diagnosis of the pathogen.[1] Enzyme-linked immunosorbent assay (ELISA) is recommended in dairy cattle with high prevalence (>5%) and pooled or individual fecal culture or polymerase chain reaction (PCR) for dairy cattle with low prevalence (<5%).[8] In

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a systematic review, published in 2017, regarding the prevalence of paratuberculosis in Latin America,[9] no prevalence data were reported for Ecuador, Cuba, Guatemala, Dominican Republic, Nicaragua, Honduras, El Salvador, Costa Rica, and Paraguay. Furthermore, in another more recent study, from 2019, for Ecuador, no confirmation of the presence of MAP in any specie had been reported, and no official paratuberculosis control program is in place in this country.[10] Sporadically, Ecuador has reported the presence of paratuberculosis in its territory in reports to the OIE-World Organization for Animal Health (Animal Health Information Database, 2013). However, there is no information of how the diagnosis was done, and MAP, as far as we know, has never been isolated in culture in the country. This may be due to the lack of knowledge concerning the disease by the livestock sector and/or the veterinarians in Ecuador, and also the absence of a specific control program, the limited availability of diagnostic tests (indirect ELISA [iELISA]), and the lack of culture facilities. The present study was realized to obtain information about the prevalence of paratuberculosis in three different regions of Ecuador using a commercial ELISA and confirm the presence of paratuberculosis in the country with the isolation of MAP in culture medium.

Methods

Cattle herds

Three dairy cattle herds were sampled in the period between March and August 2018 in three different regions in three Ecuadorian provinces (Mejia, Patricia Pilar, and Victoria del Portete in Pichincha, Santo Domingo de los Tsachilas, and Azuay provinces) [Figure 1] for the location**.** Cattle herds with between 400 and 1200 heads were sampled and only cows between 2 and 8 years were considered for this study because paratuberculosis is characterized by a long incubation period (1.5–2 years) before cows become fecal culture positive for MAP.[11] No clinical cases of paratuberculosis were detected in the cows during the time of this study.

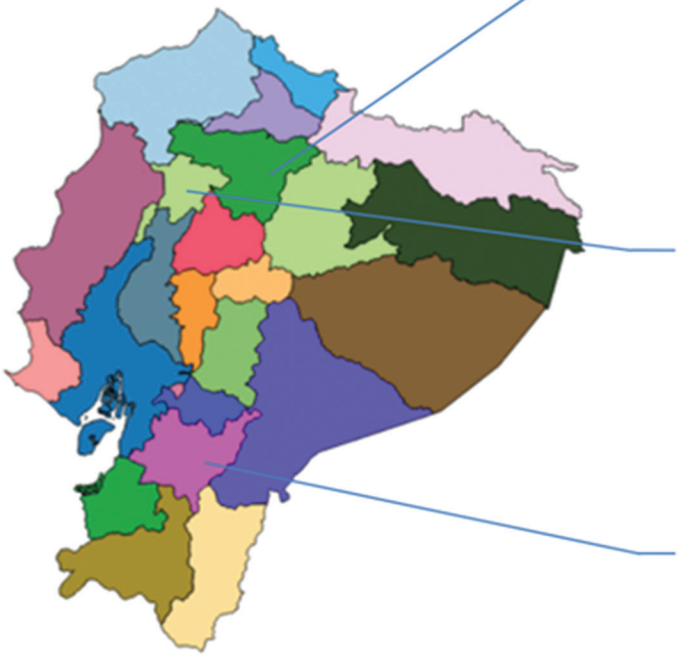
Blood samples and indirect enzyme-linked immunosorbent assay

Six hundred blood serum samples were collected, 200 samples of each cattle herd from the tail vein of the cows, and tested with a commercial indirect immunoenzymatic assay (iELISA, ID Screen® Paratuberculosis, France) to determinate the serological status of the animals. The ELISA assay was performed according to the manufacturer instructions.

Culturing of *Mycobacterium avium* subsp. *paratuberculosis*

Culture medium

Stool samples of ELISA seropositive animals were cultured in Löwestein–Jensen media (LJ) and adapted for the growth of MAP by the addition of sodium pyruvate (0.5%) and mycobactin (2 µg/ml) (ID-VET, France). Two different cocktails of antibiotics were used in the medium to overcome growth of other microorganisms: (a) PANTA with a final concentration of polymyxin B (50,000 U/ml), amphotericin B (5 µg/mL),



Mejia– Machachi*

ZN+	IS*900*+
18.3% (20/109)	4.6% (5/109)
*Seroprevalence	54.5% (109/200)

Patricia Pilar–Santo Domingo*

ZN+	IS*900*+
50% (7/14)	14.3% (2/14)
*Seroprevalence	7% (14/200)

Victoria del Portete–Azuay*

ZN+	IS900+
11.1% (3/27)	0% (0/27)
*Seroprevalence	13.5% (27/200)

Figure 1: Prevalence data according to the geographic area. 200 cows of each of the three areas were tested with an enzyme-linked immunosorbent assay, and fecal samples of seropositive cows were grown for the presence of mycobacteria. The prevalence of mycobacteria and *Mycobacterium avium* subsp. *paratuberculosis* (polymerase chain reaction positive for insertion sequence 900) in stool samples of seropositive cows was calculated

nalidixic acid (20 µg/mL), trimethoprim (5 µg/mL), and azlocillin (10 µg/mL) and (b) VAN: vancomycin (5 µg/mL), amphotericin B (5 µg/mL), and nalidixic acid (20 µg/mL).[12]

Fecal samples

Fecal samples were taken from the rectum introducing a hand with a long plastic glove in the rectum of the cow, in order to keep environmental contamination to a minimum. Samples were stored in the same glove turned inside out at 4°C until processing.

Isolation of *Mycobacterium avium* subsp. *Paratuberculosis* from fecal samples

Approximately 1 g of stool of each cow was placed in a 15 mL tube with conical bottom and homogenized by vortexing for 2 min with 6 mL of saline solution (0.9% NaCl). The tube was centrifuged for 5 min at 2000 rpm to remove the big particles and the supernatant, about 6 mL, was collected in a new tube of 15 mL. To decontaminate the samples, 6 mL of a 1.5% solution of hexadecylpyridinium chloride (HPC) (final concentration 0.75%) was added to this suspension, homogenized with a vortex, and incubated for 30 min at room temperature. The tube was then centrifuged for 30 min at 4000 g, and the pellet was washed with approximately 2 mL of sterile water. Finally, the tube was centrifuged for 30 min at 4000 g, and the pellet was reconstituted with approximately 0.5 mL of sterile water. One hundred microliters of the reconstituted pellet was inoculated in four culture tubes; two tubes of LJ medium + PANTA and in two tubes of LJ medium + VAN and incubated up to 14 weeks at 37°C until growth of colonies was visible.

Identification of *Mycobacterium avium* subsp. *paratuberculosis*

All colonies grown on the solid culture medium were tested with Ziehl–Neelsen (ZN) staining to confirm the presence of mycobacteria. For the isolation of DNA, several colonies of the acid-fast bacteria were resuspended in a 1.5 ml Eppendorf tube with 100 µL TE (Tris-EDTA) and boiled for 10 min at 100°C to liberate the DNA. Five microliters of this suspension was used for PCR to detect the presence of the IS900 fragment, which is highly specific for the MAP strains.[13] The primers used for the amplification were Fw CGATCGGAACGTCGGCTGGTCAGG and RevC GATCGCCTTGCTCATCGCTGCCG and the program: 3 min – 94°C; 30 cycles of 94°C – 1 min, 62°C – 1 min, 72°C – 1 min, and 72°C – 8 min.[14] A band of 218 bp, visualized in a 2% agarose gel, was considered positive for MAP. Mycobacteria negative for this IS900 PCR were identified with PCR restriction fragment analysis (PRA). The primers used for the PCR were Tb11 (5×-ACCAACGATGGTGTGTCCAT) and Tb12 (5×-CTTGTCGAACCGCATACCCT) and the program: 3 min – 94°C; 45 cycles of 94°C – 1 min, 62°C – 1 min, 72°C – 1 min, and 72°C – 10 min. For the *Bst*EII and *Hae*III digestion, 10 µL of the PCR product was used for each digestion, and the digestion products were analyzed on a 3.5% agarose gel. The fragment lengths were determined with a 50 bp ladder as reference.[15]

Statistical analysis

All analyses were performed in R statistic project.[16] R Foundation for Statistical Computing, Vienna, Austria.

Results

Of the 600 tested cows, 150 were classified as seropositive for paratuberculosis (25%). The seroprevalence per region differed significantly from 7% to 54.5% [Figure 1].

In 66 tubes of LJ-PANTA and 88 of LJ-VAN, visible growth of microorganisms was detected after 14 weeks of incubation. All culture tubes were readable, and no tubes had to be discarded for heavy contamination. With the ZN staining, we confirmed the presence of acid-fast bacilli in 30 cows: 14 cows yielded mycobacteria in both the medium, 5 only in LJ-PANTA medium, and 11 cows only in LJ-VAN medium. Although the yield of mycobacteria for both types of culture medium was not statistically significant, with the LJ-VAN medium, we isolated 45.5% more mycobacteria (n = 25) than with LJ PANTA (n = 19). Seven isolates, each from one individual cow, were identified as MAP with IS900 PCR and the PRA technique. Three MAP strains grew only in LJ-PANTA medium, two strains only in LJ-VAN medium, and two MAB strains were isolated from both the culture media [Table 1].

Although some contamination appeared in the culture tubes after 14 weeks of incubation, 1–20 colonies of other bacteria, all culture tubes were readable after 14 weeks of incubation. Twenty-five cows yielded nontuberculous mycobacteria (NTM) and three cows had two different species in their feces. These NTM were identified with PRA [Table 2]. Fecal samples of 11 cows only yielded NTM in LJ-VAN medium, 4 only in LJ-PANTA medium, and 10 in both the culture media. The kappa analysis,[17] comparing MAP and MNT isolation versus the PANTA or VAN antibiotic cocktail, showed moderate agreement (0.58% $CI_{95\%}$ [39–76] and 0.43% $CI_{95\%}$ [1.5–84]) with no statistic differences in the growth of other microorganisms (contamination), yield of mycobacteria, and isolation of MAP.

Figure 1 shows the seroprevalence and the distribution of mycobacteria in general and MAP in the three sample areas. Significant difference in the seroprevalence of paratuberculosis (7%–54%) was found between the three geographic areas, with the highest seroprevalence in Machachi. In addition, significantly more NTM were isolated from fecal samples of a cattle herd from the Province of Santo Doming, a tropical area of Ecuador, but with the lowest paratuberculosis prevalence.

The NTM other than MAP were identified with the PRA technique, and the results can be found in Table 2. *Mycobacterium nonchromogenicum* was the most common species found in fecal samples. Three cows yielded *M. avium* subsp. *avium*. Twelve isolates, all with different PRA patterns, could not be identified because the PRA patterns were not present in the PRA-site database (http://app. chuv. ch/prasite/index.html).

Table 1: Fecal culture results for the isolation of mycobacteria and *Mycobacterium avium* subsp. *paratuberculosis* from 150 seropositive cows

	Growth of microorganisms	Contamination	AFB (ZN staining)	MAP (IS*900* PCR)
LJ-PANTA	44% (66/150)	31.3% (47/150)	12.7% (19/150)	3.3% (5/150)
	$CI_{95\%}$(35-51)	$CI_{95\%}$(24.1-39.5)	$CI_{95\%}$(7.9-19.3)	$CI_{95\%}$(2.8-17.5)
LJ-VAN	58.7% (88/150)	42% (63/150)	16.7% (25/150)	2.7% (4/150)
	$CI_{95\%}$(50-66)	$CI_{95\%}$(34.1-50.3)	$CI_{95\%}$(11%-23)	$CI_{95\%}$(1.47-11.9)

Every fecal sample, after a decontamination step, was inoculated in two tubes of modified LJ medium with two different antibiotic cocktails (PANTA or VAN). MAP: *Mycobacterium avium* subsp. *paratuberculosis*, CI: Confidence interval, AFB Acid-fast bacilli, ZN: Ziehl-Neelsen, PCR: Polymerase chain reaction

Table 2: Nontuberculous mycobacteria (*n*=28) isolated from fecal samples of seropositive cows

Number	Specie	*BstE*II	*Hae*III	Host*	References
6	*M. nonchromogenicum* type 1	235/120/85	145/60/55	Dairy cattle	[16,17]
1	*M. gordonae* type 7	235/120/100	155/110/0	Dairy cattle/poultry	[18,19]
1	*M. genavense* type 1	320/115/0	125/105/0	Dairy cattle/pet birds	[20,17]
1	*M. gadium* type 1	440/0/0	175/90/60	Humans	[21]
2	*M. avium* subsp. avium type 1	235/210/0	130/105/0	Veal/pigs	[22,23]
2	*M. fortuitum* type 3	235/120/85	135/90/85	Dairy cattle	[17]
1	*M. avium* subsp. avium type 2	235/210/0	130/105/60	Poultry	[24]
2	*M. gordonae* type 5	250/225/0	130/115/0	Dairy cattle/poultry	[18,19]
12	*Mycobacterium* spp.	NA	NA		

*Host: The animal species reported previously with an NTM isolate from feces samples. NTM: Nontuberculous mycobacteria, NA: Not available, M. *nonchromogenicum: Mycobacterium nonchromogenicum*, *M. gordonae: Mycobacterium gordonae, M. genavense: Mycobacterium genavense, M. gadium: Mycobacterium gadium, M. fortuitum: Mycobacterium fortuitum, M. avium: Mycobacterium avium*

Discussion

This is the first report of the presence of MAP in Ecuador. The occurrence of MAP in dairy cattle in Ecuador was confirmed with culture and molecular identification. The presence of MAP in Latin American countries was reported for the first time about 100 years ago,[18] but the continent has a poor data collection record for paratuberculosis, and for most countries, there are no official prevalence rates and the majority of the countries do not have control programs.[10]

Prevalence in Ecuador

Three cattle herds from three different provinces in Ecuador were tested with the ID-Vet ELISA, and all three herds were infected with an overall seroprevalence of 25% with significant differences in seroprevalence for the three cattle herds. In comparison, it has been estimated that globally, in about half the countries, more than 20% of herds are infected and that the prevalence in a herd frequently exceeds 40%.[10] The ID-VET-ELISA was chosen for the diagnosis as it has demonstrated the highest overall accuracy of four commercial ELISA kits with a specificity of 99% and the diagnostic sensitivity of 31,6%.[19] Therefore, most of the seropositive cows tested in this study can be considered positive for paratuberculosis. Sensitivity of all available commercial ELISA tests is relatively low. The paratuberculosis iELISA has a preincubation step with *Mycobacterium phlei* antigens, a crude suspension of soluble antigens of a fast-growing, saprophytic *Mycobacterium* species that is widely distributed in soil and dust and on plants. This preincubation is used to remove all nonspecific antibodies in the serum and increase the specificity and test accuracy of the ELISA. However, this preincubation step compromises the sensitivity of the iELISA as also specific antibodies against MAP can cross-react in this preincubation step.[20] Considering this low sensitivity of the commercial iELISAs in our study, the real prevalence of paratuberculosis in our herds could be a factor 2 higher as we have determined.

Mycobacterium avium subsp. *paratuberculosis* culture

Before culturing of MAP, a chemical decontamination is applied to the fecal samples to eliminate all nontarget microorganisms. We used HPC as the decontaminant[21,22] which is recommended because the HPC method decontamination method yields the highest percentage recovery of MAP.[23] Culture is considered the confirmatory test for the diagnosis of paratuberculosis, and the identification of ZN-positive culture isolates is usually done with an IS900-PCR, detection of DNA insertion element in the genome, and specific for MAP.[22] The testing of fecal samples of 150 seropositive cows by culture yielded only seven MAP strains, as confirmed with IS900 PCR. Our study shows the limitation for culturing as a conformational test of the presence of paratuberculosis in cattle herds as no clinical cases of paratuberculosis were detected during this study. The low yield of MAP in our study may be due to most cows in a subclinical stage, with a low quantity and intermittently excreting MAP in fecal matter.[24] Furthermore, our culture methods could have an impact on the isolation of MAP. Different protocols recommended for culturing of MAP have different analytical sensitivities,[25] and the yield of MAP depends on type of culture medium, incubation time, and the decontamination method. The major strains of MAP (types I,

II, and III) have different needs for their growth. As stated in a reference,[26] an optimal solid culture medium for the isolation of MAP is modified Middlebrook 7H10 agar.[27] Another publication recommends LJ medium supplemented with pyruvate and mycobactin for isolating strains I and III, whereas for strains type II, it is the Herrold's egg yolk medium.[22] It also has been reported that pyruvate can be inhibitory to some isolates. Because the type of strain cannot be known in advance of culture, in this study, we only used supplemented LJ medium, but the recommendation for the future research is the use of the four solid media.[24] Concerning liquid culture medium for the isolation of MAP, this is considered the most sensitive antemortem test for the diagnosis in ruminants.[21,25] However, this medium is not available in our laboratory and has a disadvantage: high contamination rates of the culture medium. Growth can be detected sooner using liquid culture techniques, but the identification of the organism is more difficult in liquid culture medium because colony morphology is not visible, and the growth of other organisms needs to be distinguished.[27] Concerning culture time, we cultivated only for 14 weeks, and the prolongation of the incubation period, especially for solid medium to more than 6 months increases the detection of MAP.[26]

Antibiotic cocktail VAN versus PANTA

To avoid contamination of culture media, a common occurrence in isolating MAP, the use of an antibiotic combination in the culture medium is recommended. The long incubation time of the culture tubes and the nature of the clinical samples, feces that are heavily contaminated with other bacteria, and molds make the cultures, despite a previous chemical decontamination step, prone to contamination and in general, about 5%–15% of the cultures cannot be read. In our study, all the culture tubes were readable, and no tubes had to be discarded for heavy contamination, although in about 33% of the tubes 1–20 colonies of other microorganisms were detected. However, the presence of antibiotics in the culture medium can have an inhibitory effect on the growth of mycobacteria.[25] We found no significant difference for MAP isolation with the two different antibiotic cocktails; however, the number of isolated MAP strains was low. In the media with the VAN cocktail, although statically not significant, about 32% more NTM were isolated. Apparently, some of the antibiotics in the PANTA cocktail inhibit the growth of NTM.

Nontuberculous mycobacteria other than *Mycobacterium avium* subsp. *paratuberculosis*

Regarding the NTM isolated in this study, this finding has also been reported in other studies with animals [Table 2]. The majority of the isolated NTMs are considered opportunism pathogens and have a low pathogenicity for humans but can cause diseases in the immunocompromised patient. Earlier reports have also mentioned the isolation of NTM from milk[28] and from lymph nodes[29] of otherwise healthy cattle. We cannot exclude that their presence in the intestinal tract, or in milk or lymph nodes, may evoke sensitization and triggers an immunological response in the animal causing false-positive results in the paratuberculosis iELISA. Three of our seropositive cows yielded *M. avium* ssp *avium* from the fecal samples, a species with 99% sequence homology with MAP.[30] The presence of NTMs in the cows' intestinal tract may also interfere with other diagnostic methods such as the tuberculin skin test and the interferon-γ test.[31]

Conclusions

Here, we report for the first time the prevalence of paratuberculosis in dairy cattle in Ecuador, and we register a relative high prevalence. The isolation of MAP can be a challenge and different culture mediums are available, all with a specific diagnostic yield. The sensitivity of culturing of MAP for the confirmation of infection is limited. NTM were isolated from approximately 20% of the intestinal tracts of our cattle and a specific antibiotic combination in the culture medium (VAN) yielded a 32% higher number of NTM strains from fecal samples. Our report shows the need for the implementation of a control program, and a study to confirm the presence of clinical paratuberculosis in our country and the determination of risk factors. Furthermore, the prevalence of MAP infection in the sheep and goat population of Ecuador, an important life stock, needs attention.

Limitations of this study

No control group of fecal samples was investigated in this study to determine if seronegative cows also have MAP and/or NTM in their feces. The aim of this study was to isolate MAP and determine the prevalence of paratuberculosis in three cattle herds in Ecuador. The high prevalence of NTM in fecal samples came as a surprise. Twelve NTM could not be identified with PRA analysis and will be identified in the future with sequence analysis of the *rpoB* and 16S rRNA gene. This identification is not considered of importance for this manuscript.

Ethics approval and consent to participate

According to national regulations in Ecuador, the need for ethics approval is unnecessary for sample collection for the diagnosis of farm animals ("Ley Orgánica de Sanidad Agropecuaria" 2017, Asamblea Nacional, República del Ecuador). Written informed consent was obtained from the cattle owner who was informed of the results of this investigation. Blood drawing was done by veterinarians and free of cost to cattle owners.

Consent for publication

Written informed consent was obtained from the cattle owner who was informed of the results of this investigation and authorized publication of the data.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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