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PRELIMINARY OBSERVATIONS ON *MYCOBACTERIUM* SPP. IN DAIRY CATTLE IN ECUADOR

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Abstract. This study evaluated bovine tuberculosis in Mejia canton, a major dairy cattle production region in Ecuador. Randomly selected cattle (1,012 from 59 farms) classified according to herd size were tested by the single tuberculin test (STT). Sixty days later, positive reactors were tested again by the comparative tuberculin test (CTT). In addition, tissue samples from two STT-CTT-positive reactors detected on a farm were obtained in a local slaughterhouse and analyzed bacteriologically. A total of 4.24% of the cattle were positive in the STT and 3.85% were positive in the CTT, with the highest number (7.95%) in large herds versus 3.4% in medium herds and 0.3% in small herds. *Mycobacterium bovis* was isolated from mesenteric lymph nodes and lungs of one animal. A 16S ribosomal RNA-based polymerase chain reaction confirmed culture results and differentiated mycobacteria other than *M. tuberculosis*. This study confirms the zoonotic importance of tuberculosis in Ecuadorian dairy cattle with herd size likely to be a crucial parameter in the prevalence of the disease. The implementation of a national control program is necessary and should be based on the detection of positive cattle by STT in combination with CTT.

INTRODUCTION

Bovine tuberculosis (BTB) is an important zoonotic disease that has a worldwide distribution. The disease is caused by *Mycobacterium bovis* and apart from cattle, domestic animals and wildlife can occasionally be infected.[1,2] In developing countries in Africa, Asia, Latin America, and the Caribbean, where dairy production industry is a priority, intensification of the dairy industry has favored the transmission of the disease because proper standards for comtrolling BTB are often lacking. Thus, BTB is still causing severe economic losses in livestock due to loss of production, mortality, and condemnation of carcasses.[3]

Cattle become infected mainly by respiratory route and remain asymptomatic during the first few months after infection, but symptoms can appear when the delicate balance between the host and the infectious agent is lost because of stress factors such as immunosuppression or malnutrition.[1] After infection, nodular granulomas, known as tubercles, start to develop; these can occur in any tissue but are most frequently observed in lymph nodes, lungs, intestines, liver, spleen, pleura, and peritoneum. Clinical evidence of TB is usually lacking, and symptoms, if present, are not specific, e.g., sub-febrile temperatures, coughing, fatigue, loss of appetite, and reduced milk production.[4]

The status of BTB in Ecuador is not documented or clearly quantified because of several factors. These include lack of proper recording of positive cases, limited use of diagnostic tests, and insufficient veterinary inspection in most of the slaughterhouses. Isolated surveys carried out on BTB in cattle report a prevalence of 3.91% in the northern part of the country (Andino-Ashqui, unpublished data). In 2001, 46.55 cases of human TB per 100,000 inhabitants were reported by the Ministry of Public Health.[5] Nevertheless, studies have not been conducted to quantify cases of human TB caused by *M. bovis*. In developing countries, the frequency and involvement of *M. bovis* in non-pulmonary TB is largely unknown because of limited laboratory facilities for culture and identification of tubercle bacilli.[6]

The standard method used for routine diagnosis of BTB is the tuberculin test,[7] which consists of reading the skin reaction to an intradermally injected purified protein derivative (PPD). Differentiation of the causative *Mycobacterium* species usually requires *in vitro* growth of the organism. Alternatively, the polymerase chain reaction (PCR) has been used with considerable success,[8] and has significantly reduced the time needed to confirm diagnosis in suspected cases. The purpose of this study was to evaluate the situation of BTB in Mejia canton, a major dairy cattle production region in Ecuador.

MATERIALS AND METHODS

Study design. This study consisted of two parts: a field study in 2003 in Mejia canton, located in the Pichincha Province of Ecuador, followed by microbiologic analyses in Antwerp, Belgium. The field study was composed of a primary screening by a single bovine tuberculin test (STT) intradermally in randomly selected animals from three types of farms. Positively reacting animals were tested again with bovine and avian tuberculin by a comparative tuberculin test (CTT). Follow-up of skin-positive animals to the slaughterhouse was impossible for most animals. Therefore, samples from suspected bovine organs from the same region were collected in a slaughterhouse for further laboratory testing. Analyses of field samples were conducted at the Department of Microbiology, Unit of Mycobacteriology, Institute of Tropical Medicine (Antwerp, Belgium). Laboratory studies included *in vitro* culture, microscopic analysis, and PCR.

Tuberculin skin test. A total of 1,012 randomly selected cross-bred dairy cattle improved by Holstein-Friesian crossbreeding from 59 farms were tested by an intradermal tuberculin skin test. Based on their herd size, dairy farms were grouped as large (more than 70 cattle), medium (25–70 cattle), and small (1–25 cattle). Tuberculin tests were restricted to animals more than six months of age, i.e., 22 ani-

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mals randomly selected from each large or medium farm and all animals from small farms. All animals were ear-tagged. Injection sites were cleaned and disinfected; swellings were measured with callipers immediately after inoculation and 72 hours later. A negative result in the SST was a swelling ≤ 3 mm, a suspicious result was a swelling between 3 and 5 mm, and a positive result was a swelling > 5 mm.

To confirm positive or suspicious STT reactions, a comparative intradermal tuberculin test with bovine and avian tuberculin (CTT) was carried out after 60 days. The interpretation of the CTT after 72 hours is based on the comparison between the subsequent swellings. Reactions to bovine tuberculin ≤ 5 mm and > 4 mm larger than the reaction to avian tuberculin were considered positive.

For the STT, 0.1 of mL bovine tuberculin (PPD-protein *M. bovis*, strain AN-5 at 25,000 IU/mL; CZ Veterinaria SA, Porriño Spain) was injected intradermally 7 cm from the base of the ventral side of the tail. The CTT injections were administered intradermally on the left side of the neck with the same bovine tuberculin and 0.1 mL of avian PPD tuberculin (0.5 mg/mL, 25,000 IU/mL; Servicio Nacional de Sanidad, Buenos Aires, Argentina) in previously shaven injection sites 12 cm apart.

Isolation of *Mycobacterium* spp. One hundred twenty-five biopsy specimens (lymph nodes, lungs, kidneys, and mammary glands) were taken from 40 cattle in the local district slaughterhouse with suspicious macroscopic lesions. In addition, 11 and 18 necropsy samples, respectively, were obtained from two cattle that were slaughtered (no. 65 and no. 1555) and positive in the CTT.

All specimens were stored in 2-mL Eppendorf (Hamburg, Germany) tubes containing Dubos broth (no. 0385-17-6; Difco Laboratories, Detroit, MI) supplemented with PANTA (Bactec PANTA Plus Kit no. 440 476 4; Becton Dickinson, Franklin Lakes, NJ) to preserve the mycobacteria and retard growth of contaminants.[9] Samples were transported at 5°C and stored at –20°C at the Department of Microbiology, Unit of Mycobacteriology, Institute for Tropical Medicine in Antwerp until processed.

***In vitro* culture and phenotypic identification.** All samples were decontaminated using the inverted Petroff method, i.e., 20-minute decontamination with 1 N HCl followed by alkaline neutralization (1 N NaOH).[10] Subsequently, Ziehl-Neelsen staining and inoculation into Löwenstein-Jensen and Stonebrink[11] media was conducted. *In vitro* identification of mycobacteria was carried out using biochemical (i.e., niacin production, nitrate reduction, urease production) and inhibition tests (thiophene-2-carboxylic hydrazide) and pigmentation and colony morphology as described by Lévy-Frébault and Portaels.[12]

Polymerase chain reaction. Necropsy samples from the two CTT-positive cattle were examined by PCR. A nested PCR was performed as described by Portaels and others[13] with primers for the 16S ribosomal RNA gene.[14] Primers P1: 5′-TGCTTAACACATGCAAGTCG-3′ and P2: 5′-TGAGAT TTCACGAACAACGC-3′ were used at 56°C in the first PCR as general primers for identifying the *Mycobacterium* genus. Primers P3: 5′-AACCCGGACCTTCGTCGATG-3′ and P9: 5′-CATGTCTTGTGGTGGAAAGCGC-3′ were used at 66°C in the second PCR for specific identification of the *M. tuberculosis* complex. The final PCR product was approximately 540 basepairs.

DNA was extracted from tissues as reported by Portaels and others[9] using proteinase K. Briefly, 50 μL of proteinase K (no. V3021, 20 mg/mL; Promega, Madison, WI) and 250 μL of lysis buffer (1.6 M guanidine hydrochloride, 60 mM Tris, pH 7.4, 1% Triton, 60 mM EDTA, 10% Tween-20) were added to the decontaminated samples (250 μL) and incubated for one hour at 60°C with shaking (200 rpm). Approximately 80 mg of glass beads was then added, samples were sonicated for five minutes at room temperature in a water bath sonicator (14 kHz; Branson 1200; Branson Ultrasonics Corporation, Danbury, CT), 40 μL of acidified diatomaceous earth solution was added, and the suspensions were incubated for a maximum of two hours at 37°C with shaking (200 rpm). The suspensions were extracted twice with 70% ethanol and once with acetone with intermediate short centrifugations. The pellets were dried at 50°C in a heating block (Dri-Bath 16500; Thermolyne, Merck-Belgolabo, Belgium) and resuspended in 90 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8), followed by incubation for 20 minutes at 55°C while shaking to obtain complete homogenization. The sample was briefly centrifuged and 50 μL of supernatant was transferred to a new tube.

For the first PCR, 10 μL of the DNA extract was added to 40 μL of PCR mixture containing 50 pmol of each primer, 1 unit of Ampli *Taq* DNA polymerase (no. M1668; Promega), 200 μM dNTP (no. 27-2094-02; Pharmacia, Uppsala, Sweden), 25 μL of buffer, and 7 μL of milli-Q water (Millipore, Billerica, MA) and overlaid with two drops of mineral oil (no. 50138; ICN, Costa Mesa, CA). A negative control (milli-Q water) and positive control (DNA at a specific concentration) were included in each PCR. Denaturation was at 94°C for 5 minutes, amplification for 40 cycles at 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds, and final extension at 72°C for 10 minutes.

For the second PCR, 1 μL of the first PCR product was amplified in a 25-μL reaction mixture containing 25 pmol of each primer, 0.5 units of Ampli *Taq* DNA polymerase, 200 μM dNTP, 12.5 μL of buffer, 8.5 μL of milli-Q water and overlaid with one drop of mineral oil. Amplification was composed of 25 cycles as described for the first PCR with an annealing temperature of 66°C. A total of 75 μL of amplified DNA plus 2.5 μL of loading buffer (Fermentas, St. Leon-Rot, Germany) and a molecular size marker were subjected to electrophoresis on a 2% agarose gel (no. EP-0010-10; Eurogentec, Seraing, Belgium) in 0.5% TAE buffer (1 mM EDTA, pH 8.0, 40 mM Tris-acetate). Bands were detected by staining with ethidium bromide (Bio-Rad Laboratories, Hercules, CA) and transillumination with ultraviolet light. The pattern of bands obtained was compared with those of negative and positive controls.

RESULTS

The STT in 1,012 cattle showed 44 positive reactors (4.34%) distributed on large (26), medium (17), and small (1) farms, respectively, in addition to 14 suspected cases (1.38%) (Table 1). To confirm these results or discard possible false-positive results, the CTT was used 60 days after the STT and was conducted only with positive and suspected positive animals.

The CTT detected 39 positive cases among the STT-positive reactors. All five false-positives results were from

TABLE 1
Results of the tuberculin test of 1,012 cattle in Mejia, Ecuador*

Type of farm	No. of animals	No. of STT-positive animals (%)	No. of STT suspected animals (%)	No. of CTT-positive animals	No. of TB-positive farms/ total no. of farms
Large	327	26 (7.95)	7 (2.14)	26 (7.95)	6/15
Medium	353	17 (4.82)	4 (1.13)	12 (3.40)	2/16
Small	332	1 (0.30)	3 (0.90)	1 (0.30)	1/28
Total	1,012	44 (4.34)	14 (1.38)	39 (3.85)	9/59

* STT = single tuberculin test; CTT = comparative tuberculin test; TB = tuberculosis.

medium-sized farms (Table 1). These animals showed a strong reaction against both bovine and avian tuberculin, but the avian reaction was greater than the bovine reaction. All suspicious cases in the first test were negative in the second confirmative test. Thus, 3.85% of the animals were positive by tuberculin skin test.

Of 125 biopsy specimens obtained from the slaughterhouse, only five samples (from five different animals) yielded positive cultures. *In vitro* development was slow, i.e., it took 8–21 weeks before growth was visible and all cultures yielded exclusively mycobacteria other than *M. tuberculosis* (Table 2). None of the samples were positive by Ziehl-Neelsen staining.

Mycobacterium bovis was isolated from 4 of 29 necropsy samples from the two cattle that showed positive reactions in the CTT (Table 3). Ziehl-Neelsen staining of these samples showed mycobacteria in two mesenteric lymph nodes from both animals and in one lung.

The nested PCR for the 16S ribosomal RNA gene was conducted on the 29 specimens from two slaughtered cattle and confirmed the presence of *Mycobacterium* spp. in six samples. The PCR showed that only four organisms belonged to the *M. tuberculosis* complex (Table 3). Of three Ziehl-Neelsen–positive samples from the necropsy samples, two were identified by PCR as *M. bovis*, (both from the same animal), and the other as exclusively mycobacteria other than *M. tuberculosis* (Table 3).

DISCUSSION

In most of Latin America, the zoonotic importance of BTB is not well quantified. Notable exceptions include Argentina and in Costa Rica, where international trade has resulted in close collaboration between governments and cattle owners associations, resulting in the control and elimination of BTB.[6] In Mexico, a high prevalence (> 4% by tuberculin skin test) is similar to that observed in Ecuador (3.85%), but is higher than the prevalence in Uruguay (0.5%) reported by Gil and Samartino.[15]

In previous isolated surveys in Ecuador, the STT and the CTT demonstrated variable prevalences of BTB over time and among various provinces ranging from 0.33% in Tungurahua in 1977 to 4.92% in the Pichincha Province in 2002 (Table 4). Results within the province of Pichincha also differed markedly. A prevalence of 2.80% was observed in Cayambe canton (Andino-Asqui O, unpublished data), which is located in the same province as the region in the present study. However, the results of our study were in sharp contrast to the prevalences of 0.43% (Torres L, unpublished data) and 0.47% (Salazar JC, unpublished data) in a survey of 18 herds in the same area. The 3.85% positive reactors found in the present study are consistent with the 4.92% obtained from 3,089 cattle sampled in Mejía canton on 13 large farms (Cano G, unpublished data).

The differences in prevalence found in relation to herd size are surprising (i.e., 7.95% and 3.40% on large and medium farms, respectively, and surprisingly only 0.3% on small farms). Although the limited number of animals investigated in this study does not allow firm conclusions to be made, higher risks for BTB in commercial (large) than in traditional (small) farms might be explained by closer contact between animals in the larger farms. Herd size as a decisive factor merits further investigation in view of the expansion of the dairy industry in Ecuador in recent years, which was caused by the high demand for milk and milk by-products. Furthermore, differences observed by other investigators should be related to the type of farms in the respective surveys.

Adams estimated that the sensitivity and specificity of the caudal fold single were 72% and 98.8%, respectively, whereas the sensitivity and specificity of the CTT with bovine and avian PPD was between 68.6% and 95% and 88.8% and 99.9%, respectively.[16] The rate of false-negative results is influenced by the time since exposure to environmental strains, immunosuppression, or anergic reactions in the early postpartum period. Desensitization can occur because of too short time intervals between tuberculin skin tests, errors in the pro-

TABLE 2
Results of positive *in vitro* cultures from 125 biopsies collected in the slaughter house in Mejia, Ecuador*

Sample no.	Type of sample	ZN	CM	P	N	NR	Ur	TCH	Final identification
BK-03-1445	Lung	–	R	N	–	–	–	+	MAIS
BK-03-1709	Lung	–	R	N	–	–	+	+	*M. gordonae*
BK-03-1712	Lung	–	S	N	–	+	–	+	*M. szulgai*
BK-03-1452	Lymph	–	S	N	–	–	–	+	*M. celatum*
BK-03-1719	Lung	–	R	N	–	–	–	+	MAIS

* ZN = Ziehl-Neelsen; CM = colonial morphology; P = pigmentation; N = niacin production; NR = nitrate reduction; Ur = urease production; TCH = thiophene-2-carboxylic hydrazide; – = negative; R = rough; N = non-chromogenic; + = positive; MAIS = *Mycobacterium avium-intracellulare-scrofulaceum;* S = smooth.

TABLE 3

PCR-positive samples using 16S ribosomal RNA gene from two tuberculin-positive reactors in comparison with Ziehl-Neelsen and *in vitro* culture*

Animal no.	Sample no.	Type of biopsy	ZN	PCR		*In vitro* culture
				Msp	MTC	
65	BK-04-355	Lung	–	+	–	NG
65	BK-04-359	Mesenteric lymph node	–	+	+	*Mycobacterium bovis*
65	BK-04-360	Mesenteric lymph node	+	+	–	NG
1555	BK-04-410	Lung	+	+	+	*Mycobacterium bovis*
1555	BK-04-411	Mesenteric lymph node	+	+	+	*Mycobacterium bovis*
1555	BK-04-413	Mesenteric lymph node	–	+	+	*Mycobacterium bovis*

* PCR = polymerase chain reaction; ZN = Ziehl-Neelsen; Msp = *Mycobacterium* sp., MTC = *Mycobacterium tuberculosis* complex; – = negative; + = positive; NG = no growth.

cedure, e.g., injection of insufficient tuberculin, use of tuberculins of reduced potency, and test and variability among observers.[17] Therefore, in the present study as a precaution, an animal was declared positive when its reaction versus bovine tuberculin was at least 4 mm bigger than the reaction to avian PPD, which is a more rigorous criterion in comparison with the standard used by the United States Department of Agriculture.[18]

False-positive reactions may be caused by sensitizations as a result of exposure to *M. avium*, *M. paratuberculosis*, environmental mycobacteria, and skin tuberculosis caused by slow-growing mycobacteria (*M. marinum*, *M. ulcerans*, *M. kansasii*, *M. avium-intracellulare*, and *M. scrofulaceum*) and rapidly growing mycobacteria (*M. fortuitum* and *M. chelonae*[19]). Other factors include the presence of *Corynebacterium*, *Fasciola hepatica*, and some *Nocardia* species, which pose problems in several countries.[20] In cattle vaccinated against paratuberculosis, the interpretation of single tuberculin tests might be difficult, but differentiation is possible by the use of the CTT.[21] *Mycobacterium avium* has frequently been recovered from cattle, causing only non-progressive lesions in the mesenteric lymph nodes.[22] Sensitization is often caused by exposure to infected domestic or wild birds, and occasionally by exposure to pigs infected with the *M. avium-intracellulare-scrofulaceum* complex, in which animals show a high reaction to avian PPD, as was observed in five animals from medium-sized farms. Therefore, a positive result in the CCT was restricted to those reactions where bovine tuberculin caused a minimum swelling of 5 mm and differed by at least 4 mm from the swelling caused by the avian tuberculin; the same criterion was used in previous studies in Ecuador (Andino-Ashqui O, unpublished data and Cano G, unpublished data). Although STT and CTT were used in most of the previously performed surveys in Ecuador, the prevalence of aspecific reactions was not clearly mentioned and results referred to the animals positive only for bovine tuberculosis.[3]

In vitro cultures of 125 specimens from suspected cattle resulted in the isolation of mycobacteria in 5 samples (4%), all of which were mycobacteria other than *M. tuberculosis*. Thus, suspected postmortem lesions are not necessarily caused by *M. bovis* as reported by Quinn and others,[23] but may be caused by another pathogenic mycobacteria. In a survey in Argentina, bacilli in cattle from slaughterhouses were identified as *M. bovis*, *M. gastri*, *M. flavescens*, *M. phlei*, and *M. triviale*.[24] In Burundi, Rigouts and others isolated *M. bovis* from 15 of the 82 cattle sampled; 78% of those isolated grew on Stonebrink medium, which showed the preference of *M. bovis* for this medium.[25] Also identified were mycobacteria other then *M. tuberculosis* (in descending order of frequency: *M. terrae*, *M. nonchromogenicum*, *M. intracellulare*, *M. gordonae*, *M.* sp. rapid grower, and *M. paratuberculosis*).

The isolation of *M. bovis* from only 4 of the 29 samples from two positive reactors with a high positive response in the tuberculin test demonstrates the difficulty related to the *in vitro* culture of this species because the distribution of the bacilli in lesions is not homogeneous.[10] Another factor that could have contributed to this is the stage of the disease at the time of sampling. Therefore, this result shows the need for a

TABLE 4

Results of previous surveys on bovine tuberculosis in Ecuador (1977–2003)*

Authors†	Year	Province	Test	No. of animals tested	% Positive for bovine tuberculosis
Acosta	1977	Tungurahua	STT-CTT	2,132	0.33
Torres	1996	Pichincha	STT	4,888	0.43
Salazar	2002	Pichincha	STT-CTT	3,006	0.47
Andino-Ashqui	2001	Pichincha/Imbabura/Carchi	STT-CTT	178	2.80
				329	7.29
				516	2.13
				1,023 (total)	3.91 (total)
Cano	2002	Pichincha	STT-CTT	3,089	4.92
Burbano	2002	Carchi	STT-CTT	3,011	1.73
Bedón	2003	Imbabura	STT-CTT	3,005	2.43
Alemán	2003	Tungurahua	STT-CTT	4,012	1.22

* STT = single tuberculin test; CTT = comparative tuberculin test.
† Data are from doctoral theses.

detailed investigation to avoid false-negative cases. Furthermore, it has been shown that freezing-thawing cycles decrease the viability of mycobacteria.[26]

Although direct identification of acid-fast organisms by microscopy is fast, it does not identify the *M.* species.[27] In the present study, characteristics that identify *M. bovis*, such as acid-fast staining, rough morphology of the colony, susceptibility to thiophene-2-carboxylic acid hydrazide, and the absence of niacin production,[25] showed the usefulness of *in vitro* identification, especially when the PCR is not feasible. Microscopic examination by Ziehl-Neelsen staining in 125 specimens from the slaughterhouse and in 29 specimens from the two positives reactors showed the bacilli in only three samples. The low sensitivity of this method is well-known[14] because large numbers of organisms ($> 10^4$/mL) must be present to make detection reliable.

All positive results obtained by conventional methods have been confirmed by PCR. The advantage of the PCR is that these results were obtained after 48 hours, whereas *in vitro* identification required 6–8 weeks. In addition, the PCR can determine whether the organisms belong to the *M. tuberculosis* complex, which requires another week by conventional methods. Since less optimal conditions during transport may have adversely affected the culture results, analysis immediately after sample collection is recommended.

The prevalences observed in the present study confirm the importance of bovine tuberculosis in Mejía canton and stress the need for surveillance at the national level. Use of diagnostic tools such as intradermal tests, *in vitro* cultures, and PCR will be useful in identifying reactors in herds, increasing epidemiologic information, and differentiating causative species. These tools will increase understanding of the zoonotic consequences, including the possible relation with herd size, and help to implement the most efficient control measures. Whether the PCR as a diagnostic tool is the most useful technique for rapid detection of *M. bovis* will require further evaluation on a larger sample size. In Ecuador, this technique is still too expensive for routine use. Thus, the STT and CTT in the field and use of *in vitro* cultures in the laboratory to identify the cause of BTB within a herd is recommended.

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