Preventive Veterinary Medicine 101 (2011) 65–72

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Post-mortem examination and laboratory-based analysis for the diagnosis of bovine tuberculosis among dairy cattle in Ecuador

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ARTICLE INFO

Article history:
Received 6 July 2010
Received in revised form 26 April 2011
Accepted 29 April 2011

Keywords:
Veterinary inspection
In vitro culture
PCR
Mycobacterium bovis

ABSTRACT

Veterinary inspection in slaughterhouses allows for the detection of macroscopic lesions reminiscent of bovine tuberculosis, but the presence of *Mycobacterium bovis* must be confirmed by laboratory methods. This study aimed at comparing the performances of the standard diagnostic tools used to identify *M. bovis* in tissue specimens sampled from suspicious animals. During a two years period, 1390 cattle were inspected at the Machachi abattoir in the Mejia canton – Ecuador. A total of 33 animals with granulomatous lesions were detected, representing 2.33% (16/687) and 2.42% (17/703) animals examined in 2007 and 2008, respectively. Ninety-four tissue specimens were sampled and screened for the presence of mycobacteria. Acid-fast bacilli were identified in one third of the suspicious cattle (11/33) and suggestive microscopic lesions in 27.3% (9/33) of the samples examined by direct microscopy and histopathology, respectively. Culturing on Stonebrink medium and 16S-rRNA-based polymerase chain reaction (PCR) yielded 36.4% (12/33) and 27.3% (9/33) of positives, respectively. Compared to culture, other diagnostic procedures displayed a lower sensitivity, with 56.5% for PCR, and 43.5% for direct microscopy and histopathology; however, the specificity was higher (94.4% for PCR and microscopy, and 97.2% for histopathology). We conclude that reliable post-mortem laboratory testing either requires the combination of a set of available diagnostic tools or necessitates the development of improved new-generation tools with better sensitivity and specificity characteristics.

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doi:10.1016/j.prevetmed.2011.04.018

1. Introduction

Bovine tuberculosis (BTB) is present in most developing countries where surveillance and control activities are not or inadequately implemented (Cosivi et al., 1998). In Ecuador, there is no BTB control program in place. An apparent prevalence of 7.95% was observed among dairy cattle from large herds in 2004 (Proaño-Perez et al., 2006), and it increased to 8.63% three years later in the same region (Proaño-Pérez et al., 2009). Herd size was identified as an important risk factor in this husbandry system, and the number of skin-test-positive cases also increased significantly with cattle age (Proaño-Pérez et al., 2009).

BTB control programs consist of pasteurization of milk, meat hygiene control through macroscopic inspection at slaughter (veterinary inspection), and skin testing to detect and cull infected animals (Collins, 2006).

Cattle are mainly infected with *M. bovis* through inhalation of aerosolized droplets (Goodchild and Clifton-Hadley, 2001). However, the process by which exposure to infection can lead to a range of disease outcomes is not fully understood (Neill et al., 1994; Pollock et al., 2006). Not all infected cattle display gross lesions, but if present, they are often located in thoracic lymph nodes (Whipple et al., 1996) even in apparently healthy cattle. Lesions due to non-tuberculous mycobacteria (NTM) can be easily mistaken for BTB lesions (Oloya et al., 2007). Consequently, laboratory approaches are important to confirm and identify the mycobacterial species involved.

Different methods can be used to identify the disease in humans and animals. Microscopic detection of mycobacteria is insensitive and does not permit the identification of the mycobacterial species involved. Culture of *M. bovis* can be more sensitive and allows for species identification, but is time consuming due to the slow growth of the bacilli (Mangiapan et al., 1996). Polymerase chain reaction (PCR) can reduce the time for detection and also identify the species.

The aim of this study was to compare currently available laboratory tools to diagnose BTB using abattoir samples taken during veterinary inspection, characterize the distribution of lesions observed in the organs of inspected cattle, and study the true prevalence of BTB.

2. Materials and methods

2.1. Study design

Veterinary inspection was carried out in 1390 slaughtered cattle from 112 different dairy farms at the abattoir of Machachi located in the Mejia canton (0°27′SL and 78°25′WL), located in the major dairy cattle production area in northern Ecuador. There was no previous studies on BTB in this abattoir. In total, 29 interventions were performed at the slaughterhouse during the period from February to March in the years 2007 and 2008. Detailed post-mortem examination was performed in 687 and 703 cattle, respectively. In this study, all cattle were inspected during each intervention, and cattle originating from areas outside the Mejia canton were excluded. Individual data were recorded for each animal by the use of a questionnaire with a focus on age, breed, sex, name of the owner, origin of the animal and lesions found during the inspection. Five of the animals examined during the abattoir survey were previously identified as bovine comparative intradermal tuberculin test (CITT) reactors in a survey among dairy cattle in the Mejia canton (Proaño-Pérez et al., 2009).

2.2. Post-mortem examination

Detailed veterinary inspection was carried out on all cattle. The lungs, liver, spleen, kidney and mammary gland were palpated carefully and inspected externally and internally. Mandibular, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and supramammary lymph nodes were sliced into thin sections (2–3 mm) and inspected *in situ* for detection of visible lesions.

2.3. Samples

Tissue specimens were taken from multiple organs using disinfected knives (i.e., chlorine 10%) from all suspected animals for further laboratory-based diagnosis. We included all organs with visible lesions, and added lung tissue taken from the inferior lobe in all sampled cattle, even if no macroscopic lesions were visible. In total, 94 tissue samples were collected from 33 suspected cattle (2–8 specimens/animal, depending on the lesions found); 65 of them were sampled from tissues with visible gross lesions suggestive for mycobacterial infection, and the rest were samples without lesions taken from lungs.

All samples were collected in individual sterile tubes, stored in a cool box, and transported the same day to the Laboratory of Microbiology at the International Centre of Zoonoses, in Quito. Upon arrival, processing of samples was carried out using aseptic techniques in a biosafety cabinet to avoid cross-contamination between samples. In order to perform microscopy, PCR, and *in vitro* culture, approximately 5 g of tissue were stored at −20 °C in 2 mL Eppendorf® tubes containing Dubos broth (No. 0385-17-6, Difco Laboratories, Detroit, USA) supplemented with PANTA (Bactec PANTA plus kit No. 440 476 4; Becton Dickinson, NJ, USA) to inhibit the growth of contaminants and to preserve the mycobacteria. During the international transport to Belgium the samples were packed in a cooler. All samples underwent 2 freeze–thaw cycles due to the storage and shipping prior to their final processing at the Mycobacteriology Unit, in the Institute of Tropical Medicine of Antwerp, Belgium. Duplicate samples that were stored in Ecuador had only one freeze–thaw cycle and were processed locally once the culture technique was implemented.

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In addition, about 1 cm^3 of each sample was stored in 10% formalin for histopathologic analyses at the Department of Morphology and Pathology from the Faculty of Veterinary Medicine in the University of Liege, Belgium.

2.4. Microscopy and in vitro culture

Upon arrival in Antwerp, all samples for bacteriological examination were cut and homogenized in a sterile mortar with 2 mL of sterile phosphate buffered saline. *In vitro* culture was carried out after decontamination using the "reversed" Petroff method (Palomino and Portaels, 1998): 20 min incubation with 3 mL of 1 N HCl followed by neutralisation with 3 mL of 1 N NaOH and centrifugation for 20 min at 3000 × *g*. The pellet was mixed with 2 mL sterile distilled water and inoculated onto Löwenstein–Jensen (LJ) (Parks, 1997), and Stonebrink medium (i.e. LJ without glycerol but supplemented with 0.4% sodium pyruvate). Isolates were identified as *M. bovis* by spoligotyping characterized by a typical lack of spacers 3, 9, 16 and 37–42 (Kamerbeek et al., 1997). For Ziehl–Neelsen (ZN) staining and subsequent microscopic inspection to detect acid-fast bacilli (AFB), a slide was prepared using 1 drop of the processed specimen. Stained slides were examined with a normal light microscope using objective 100× and immersion oil.

2.5. Polymerase chain reaction

DNA was extracted from the re-suspended pellet using the method developed by Mangiapan and colleagues (1996). A routinely-applied, nested PCR targeting the 16S ribosomal RNA gene was performed to detect DNA specific for the *M. tuberculosis*-complex using the following primers (Portaels et al., 1996): P1 (5′-TGCTTAACACATGCAAGTCG-3′) and P2 (5′-TCTCTAGACGCGTCCTGTGC-3′) for the first run, and P3 (5′-AACCCGGACCTTCGTCGATG-3′) with P9 (5′-CATGTCTTGTGGTGGAAAGCGC-3′) for the second run. Samples were considered positive to bovine tuberculosis when a band of 500 bp was observed on a 2% (w/v) agarose gel.

2.6. Histopathology

Slides for histopathology examination were prepared using standard techniques, and processed for standard hematoxylin–eosin (HE) and ZN staining. A sample was considered positive if lesions characteristic for BTB were demonstrated (granulomatous inflammation associated with focal caseous necrosis or mineralization), or if AFB were observed in the ZN stain (Whipple et al., 1996).

2.7. Statistical analysis

The true prevalence (*TP*) based on detection of gross visible lesions was calculated using the Rogan-Gladen's Equation (1978) under a Bayesian modelling approaches (Berkvens et al., 2006). The Rogan-Gladen equation describes the estimation of *TP* through the apparent prevalence (*P′*), and the *Se*, and *Sp* of a test $[P' = TPSe + (1 - TP)(1 - Sp)]$. Since there is no gold standard test, *TP* must be estimated imposing constraints on the parameters (Berkvens et al., 2006). Bayesian approach lets to incorporate external information by specifying prior distributions on the parameters, i.e., prior knowledge and/or beliefs regarding to *Se* and *Sp* of necropsy (Enoe et al., 2000; Branscum et al., 2005). This approach makes possible inferences about *Se* and *Sp* with 95% probability interval (95% PI) of necropsy used in this study. The prior distribution on *Se* was elicited from a previous study that considers the most likely value (modal), thus the *Se* of necropsy was 0.8 with a 10th percentile of 0.55; which is acceptable for this test and was chosen because a similar procedure during the necropsy was performed in the same lymph nodes (Norby et al., 2004), and based on the same measures through veterinary inspection to discard carcasses in Ecuador (Personal communication). The prior distribution for the *Sp* was elicited from the experience of veterinarian inspectors from the slaughterhouse of the Mejia canton, taking into account the presence of other infectious diseases causing lesions in lymph nodes specially in dairy cattle, thus a modal value of 0.95 with a 5th percentile of 0.80 were set to model the uncertainty about *Sp*. The *BetaBuster* software (available at http://www.epi.ucdavis.edu/diagnostictest/) (Branscum et al., 2004) computed the two parameters of a *Beta* prior distributions based on the mode and one percentile for *Se* and *Sp*, i.e., *Beta* for *Se* (5.28, 2.07), and *Beta* for *Sp* (21.20, 2.06). A binomial model was built and Bayesian estimation was done through Gibbs sampling in WinBUGS (Spiegelhalter et al., 1996). A burn-in phase of 1000 iterations was used and the model was run for another 10,000 iterations to obtain estimates. The outcomes were mean and percentiles (i.e., 2.5 and 97.5) sampled from the posterior distributions of *TP*, *Se* and *Sp*. Three chains starting in different values were set and their convergence was analyzed through trace plot. Additionally the effect of different prior distributions for the parameters of the model was analyzed in order to evaluate how prior beliefs could affect the posterior estimates of *TP*, *Se* and *Sp*. Seven scenarios were built assuming prior different beliefs about the parameters (Table 1): (Scenario 1) No prior constraints: *TP* ∼ Uniform (0, 1), *Se* ∼ Uniform (0, 1) and *Sp* ∼ Uniform (0, 1); (Scenario 2) Prior distributions for *Se* and *Sp* uniformly distributed according to ranges suggested by Norby et al. (2004), and expert opinion of veterinaries in slaughterhouses *TP* ∼ Uniform (0, 1), *Se* ∼ Uniform (0.51, 0.98) and *Sp* ∼ Uniform (0.80, 0.90); (Scenario 3) True prevalence constrained uniformly to values less that 10% and *Se* and *Sp* with similar values to previous experiences *TP* ∼ Uniform (0, 0.10), *Se* ∼ Uniform (0.50, 1.00) and *Sp* ∼ Uniform (0.70, 1.00); (Scenario 4) True prevalence constrained uniformly to values less that 5% and *Se* and *Sp* with similar values to previous experiences but widen: *TP* ∼ Uniform (0, 0.05), *Se* ∼ Uniform (0.40, 1.00) and *Sp* ∼ Uniform (0.60, 1.00); (Scenario 5) Assuming that *Se* of the necropsy is 70% and *Sp* 80% but with high uncertainty *TP* ∼ Uniform (0, 1.00), *Se* ∼ Beta (8.00, 4.00) and *Sp* ∼ Uniform (9.00, 2.00); Scenario 6) Assuming that *Se* of the necropsy is 70% and *Sp* 80% but with low uncertainty *TP* ∼ Uniform (0,1), *Se* ∼ Beta (71.0, 31.0) and *Sp* ∼ Uniform (86.0, 16.0); and finally (Scenario 7) *TP* ∼ Uniform (0, 1), *Beta* (5.28, 2.07) and *Beta* (21.20, 2.06) which correspond to our beliefs about necropsies.

Table 1
Estimates of parameters in the Bayesian binomial model using different prior distributions for *TP*, *Se* and *Sp* of necropsy to identify bovine tuberculosis.

Scenario	Prior distribution	Posterior mean %	IP 95%
Scenario 1	*TP* ~ Uniform (0, 1)	Did not converge	–
	Se ~ Uniform (0, 1)	Did not converge	–
	Sp ~ Uniform (0, 1)	Did not converge	–
Scenario 2	*TP* ~ Uniform (0, 1)	1.11	0.005–4.69
	Se ~ Uniform (0.51, 0.98)	71.89	68.80–94.60
	Sp ~ Uniform (0.80, 0.90)	98.22	98.08–99.64
Scenario 3	*TP* ~ Uniform (0, 0.10)	1.83	0.008–4.40
	Se ~ Uniform (0.5, 1.0)	72.04	50.87–98.26
	Sp ~ Uniform (0.7, 1.0)	98.74	97.27–99.99
Scenario 4	*TP* ~ Uniform (0, 0.05)	1.95	0.01–4.50
	Se ~ Uniform (0.4, 1.0)	66.11	41.0–97.7
	Sp ~ Uniform (0.6, 1.0)	98.71	97.7–99.8
Scenario 5	*TP* ~ Uniform (0, 1)	1.50	0.005–4.30
	Se ~ Beta (8.0, 4.0)	63.63	34.63–87.83
	Sp ~ Beta (9.0, 2.0)	98.31	97.05–99.65
Scenario 6	*TP* ~ Uniform (0, 1)	0.3	0.01–1.25
	Se ~ Uniform (71, 31)	69.28	60.01–78.03
	Sp ~ Uniform (86, 16)	96.86	95.00–97.7
Scenario 7	*TP* ~ Uniform (0, 1)	1.50	0.005–4.69
	Se ~ Uniform (5.28, 2.07)	67.02	68.8–94.6
	Sp ~ Uniform (21.20, 2.06)	98.34	97.08–99.64

The agreement among the different laboratory-based diagnostic methods applied in this study was assessed by Cohen *kappa* statistic under cluster sampling (*svykappa* function) under R environment, described by Lumley (2004), which is the approach used to asses different tests without assuming that one is the best. The results obtained were classified according to the Altman scale to give a grade of significance (i.e., >0.80: very good agreement, 0.61–0.80: good agreement, 0.41–0.60: moderate agreement, 0.21–0.40: fair agreement, and ≤0.20: poor agreement) (Abraira, 2000). In addition, the Se and Sp of histopathology, microscopy, and PCR were calculated taking the *in vitro* culture as a gold standard test. The 95% confident intervals (CI) were estimated for Se and Sp of each test.

3. Results

The apparent prevalence of gross visible lesions found by veterinary inspection was 2.33% (95% CI = 1.37–3.73) and 2.42% (95% CI = 1.41–3.84) for the 2007 and 2008 surveys respectively. The *TP* was calculated to be 1.50% (95% PI = 0.005–4.69) taking into account our prior believes related with the characteristic of this test. The estimated *Se* and *Sp* of necropsy in this study were 67.02% (95% PI = 29.39–94.58) and 98.34% (95% PI = 97.08–99.64), respectively. The estimates of the *TP* did not show important differences under several constrains, they were around 1.5%, although when constrains had low uncertainty (scenario 6) the estimates of *TP* was lower. The estimates for *Sp* of necropsy were in general consistent and high. The estimates for *Se* of necropsy varied according to the constrictions and wide intervals were found (Table 1).

Macroscopically, we observed yellowish granulomatous tubercles in mediastinal (51.32%), tracheobronchial (23.68%), hepatic (11.84%), and retropharyngeal (9.21%) lymph nodes. Only one animal showed visible lung lesions. Also, the five CITT reactors showed visible lesions in thoracic lymph nodes, but not in the lungs.

From the 94 specimens, 31 yielded one or more positive results in the laboratory (Table 2): all 24 positive lymph nodes from 15 cattle showed visible lesions (8 bronchial, 7 mediastinal, 3 hepatic, 3 mammary, 2 retropharyngeal, and 1 scapular lymph node), versus only one of the 7 (14.28%) positive lung tissues (Table 2). Fourteen of 31 specimens yielded a positive result in only a single test, 6 scored positive in 2 tests, 6 in 3 tests, and 5 had a positive result in all 4 tests applied.

On the animal level, this resulted in the detection of *M. bovis* in 51.5% (17/33) of the suspected animals. In 7 animals, only one specimen was found positive, whereas the remaining animals had 2–4 positive specimens.

Histopathology examination allowed for the identification of BTB in 27.3% (9/33) of the suspected animals. It showed focal necrosis, distrophic calcification and/or Langhans- and foreign body-type giant cells in a total of 12 biopsies. Giant cells were identified in the lung sample showing gross lesions and in 3 mediastinal lymph nodes. Necrosis and/or calcification were more frequently observed (11 samples). In the remaining animals (24/33), a variety of lesions was identified in lungs (chronic interstitial pneumonia 65.7%, atelectasis 17.1%, emphysema 8.6%, granulomatous pneumonia 5.7% and bronchioloalveolar carcinoma 2.9%), and lymph nodes (caseous lymphadenitis 38.3%, follicular hyperplasia 31.9%, lymphoma 8.5%, oedema with infiltrate of macrophages 6.3%, other lesions 4.2%, and no significant lesions 10.6%).

AFB were detected in ZN-stained smears from 33.3% (11/33) of suspected animals. Fourteen (14) of 94 smears were positive: 4 from lung samples and 10 from lymph nodes, mainly bronchial and mediastinal (Table 2). However, the number of AFB detected per smear was very low, with a maximum of 10. Five cases had more than 3 AFB per slide but in 9 slides only 1 or 2 AFB were observed.

In vitro culture proved the most sensitive method in this study. *M. bovis* was isolated from 36.4% (12/33) suspected cattle. Bacilli were grown in 23 samples after about 7 weeks, and on Stonebrink medium only. The colonies were

Table 2
Distribution of positive laboratory results for bovine tuberculosis observed in 31 specimens from 17 different animals taken from dairy cattle at the slaughterhouse of Machachi, Ecuador.

Area	Animal identification	Farm	Specimen	Visible lesion	Smear ZN	Culture ST	PCR 16S rRNA	Histo-pathology
Machachi	1	A	Bronchial LN	Yes	Positive	–	–	–
	5	B	Lung	No	–	–	Positive	–
	8	C	Bronchial LN	Yes	–	Positive	–	Positive
			Retropharyngeal LN	Yes	–	–	–	Positive
	33	C	Lung	No	–	Positive		
	14	C	Retropharyngeal LN	Yes	–	Positive	–	Positive
			Bronchial LN	Yes	–	Positive	Positive	Positive
			Hepatic LN	Yes	–	Positive	Positive	–
			Mammary LN	Yes	–	Positive	Positive	–
			Lung	No	Positive	Positive	Positive	–
	23	C	Lung	No	Positive	–	–	–
			Mediastinal LN	Yes	–	Positive	–	–
	13	D	Hepatic LN	Yes	–	Positive	Positive	Positive
			Bronchial LN	Yes	–	Positive	–	–
			Lung	No	–	–	Positive	–
	26	D	Mediastinal LN	Yes	Positive	Positive	Positive	Positive
	30	D	Mediastinal LN	Yes	–	Positive	–	–
	32	D	Mediastinal LN	Yes	–	–	–	Positive
Tambillo	12	E	Bronchial LN	Yes	Positive	Positive	–	–
			Lung	No	Positive	Positive	Positive	Positive
	25	F	Mammary LN	Yes	Positive	–	–	–
Aloasi	16	G	Scapular LN	Yes	–	Positive	–	–
			Bronchial LN	Yes	Positive	Positive	Positive	–
			Mediastinal LN	Yes	–	Positive	–	Positive
	31	G	Mammary LN	Yes	Positive	–	–	–
	28	H	Bronchial LN	Yes	Positive	Positive	Positive	–
Aloag	19	I	Lung	Yes	Positive	Positive	Positive	Positive
			Mediastinal LN	Yes	Positive	Positive	Positive	Positive
			Bronchial LN	Yes	Positive	Positive	Positive	–
			Hepatic LN	Yes	–	Positive	–	–
Chaupi	20	J	Mediastinal LN	Yes	Positive	Positive	Positive	Positive

ZN = Ziehl–Neelsen, ST = stonebrink, LN = lymph node.

obtained from 19 lymph node and 4 lung specimens, all positive *M. bovis* isolates were confirmed by spoligotyping. No mycobacteria other than *M. bovis* were detected by culture.

Nested PCR detected 27.23% (9/33) positive animals. *M. tuberculosis* complex DNA was amplified in 15 of 94 samples: 10 lymph nodes and 5 lung biopsies (Table 2). Negative DNA-extraction and PCR controls yielded a negative result in all PCR runs.

Survey *kappa* analysis of the various laboratory tests revealed a substantial concordance between culture and PCR ($\kappa = 0.61$), a moderate concordance between culture and histopathology ($\kappa = 0.49$), culture and microscopy ($\kappa = 0.44$), PCR and microscopy ($\kappa = 0.47$), and PCR with histopathology ($\kappa = 0.44$) (Table 3). A low concordance was observed between microscopy and histopathology ($\kappa = 0.20$).

Compared to *in vitro* culture, the 3 remaining techniques had a low sensitivity to detect BTB. The *Se* of PCR (56.5%; 95% CI = 34.4–76.8) was higher than that of microscopy (43.5%; 95% CI = 23.2–65.5) and histopathology (43.5%; 95% CI = 23.2–65.5). Specificities were much higher with 94.4% (95% CI = 86.2–98.4) for PCR and microscopy, and 97.2% (95% CI = 90.2–99.6) for histopathology.

4. Discussion

The prevalence of BTB, as determined by the detection of gross visible lesions in slaughtered animals, was similar in 2007 and 2008 (2.32% and 2.41%, respectively). However, the true prevalence, taking into account the *Se* (83.33%) (Norby et al., 2004) and *Sp* (95%) calculated for this method, was lower (1.50%). As this study was the first of its kind in the Mejia canton, we cannot compare our

Table 3
Concordance analysis by survey sampling *Kappa* of the laboratory tests applied to diagnose BTB from specimens taken at the abattoir of the Mejia canton—Ecuador during 2007 and 2008.

Tests	Microscopy		Culture		PCR		Histopathology	
	κ value	Std error	κ value	Std error	κ value	Std error	κ value	Std error
Microscopy	1	na						
Culture	0.44	0.12	1	na				
PCR	0.47	0.15	0.61	0.08	1	na		
Histopathology	0.20	0.14	0.48	0.06	0.44	0.13	1	na

κ: *Kappa*, na: not applicable.

results with previous data of this region, even though it is the most important dairy region in the north of Ecuador. Abattoir surveys performed in other provinces showed a lower prevalence: 0.12% and 0.46% in Guayas, and 0.21% in Santo Domingo de los Sáchilas (Mata, 1973; Cueto and Suárez, 1993; Coloma, 2000). However, these studies – which were never officially published – were carried out in meat production regions and therefore are not comparable with our data from dairy cattle as shown by data from other countries. In Mexico, an abattoir survey revealed the presence of gross lesions typical for BTB in 16% of dairy cattle (Milian-Suazo et al., 2000), whereas another survey revealed significantly less gross lesions among beef cattle (0.05%) (Brown and de Anda, 1998). This difference between dairy and beef cattle has been observed in different abattoirs throughout Ecuador, and it was attributed to several factors such as cattle density, management practices and breed; however, data have never been published (personal communication). In Ecuadorian dairy industry, imported European breeds have been used to improve milk production, i.e. Holstein Friesian, which is less resistant to BTB than Zebu (Omer et al., 2001; Ameni et al., 2007; Kazwala et al., 2001).

Compared to the recently observed CITT-based prevalence data among dairy cattle from the Mejia canton (Proaño-Pérez et al., 2009), our veterinary-inspection-based prevalence was lower. This could reflect the lower sensitivity of post-mortem examination (Norby et al., 2004), implicating that a high number of animals might be classified as negative by this method. Routine abattoir inspection can be affected by the method employed, the anatomical sites examined and the stage of infection (Corner, 1994); it will fail to detect infected animals if lesions are present in tissues not being inspected. Since the veterinary inspection in our survey was done in a very comprehensive way, we believe that the early stage of infection played a role in the low detection rate. During our abattoir survey only one of the 5 bovine CITT reactors had evident gross lesions; the rest showed only minor lesions in thoracic lymph nodes. Three of the latter were identified as CITT-reactors in the year of slaughter, and therefore might be at the early stage of infection. The two remaining animals were CITT-reactors in two consecutive years and slaughtered afterwards; one of them showed characteristics of advanced disease. Nevertheless, BTB was confirmed by laboratory tests in all CITT-reactors. These findings confirm that animals only show evident characteristics in an advanced stage of the disease (Corner, 1994), although the symptoms of the disease are not pathognomonic. On the other hand, our data also confirm that *M. bovis* can be isolated from animals without gross lesions, including lungs (Whipple et al., 1996; Teklul et al., 2004).

Gross visible lesions may be caused by other organisms as well, and also NTM may constitute a confounding factor. In Tanzania, Cleaveland and colleagues (2007) identified *M. terrae, M. avium, M. chelonae, M. gordonae, M. fortuitum, M. flavescens* and *M. smegmatis* in samples from slaughtered cattle with visible lesions; however, the clinical relevance of the isolation of these environmental mycobacteria needs to be confirmed. In Ecuador, the presence of *M. avium-intracellulare-scrofulaceum, M. gordonae, M. szulgai* and *M. celatum* was reported in slaughtered dairy cattle (Proaño-Perez et al., 2006). Therefore, the identification of the species plays a crucial role in the final diagnosis of the disease. We did not isolate NTM in the present study.

The combined use of microscopy, *in vitro* culture, PCR and histopathology identified BTB in 51.5% (17/33) of the suspected animals. Nevertheless, only 6 of these animals were positive by all the laboratory methods used.

M. bovis was identified by *in vitro* culture in 36.34% of the suspected cattle. This is low compared to studies from Mexico and Argentina. In Mexico, BTB infection was confirmed by culture in 59% of cattle from 6 important dairy regions (Milian-Suazo et al., 2000), and in the Province of Santa Fé, Argentina, with 83% of samples showing visible lesions (Latini et al., 1997). The *Se* of detection by culture can be reduced due to the applied decontamination procedure or pre-analytical storage conditions of the samples, affecting the viability of the Mycobacteria (Palomino and Portaels, 1998). Freezing–thawing cycles proved to reduce the viability of mycobacteria significantly (Portaels et al., 1988). As our specimens underwent two freeze–thaw cycles, part of the bacilli may have been killed and therefore could not be isolated. AFB were detected in 14 of 94 specimens, but in 4 of them the presence of *M. bovis* could not be confirmed by culture. This might be attributed to the suboptimal storage conditions not ensuring survival of the bacteria. Nevertheless, our culture positivity rate was still higher than that observed in some other studies. In Brazil, culturing of *M. bovis* was successful in 18% of the samples (Pires de Araújo et al., 2005); no information was given on the storage conditions of the investigated samples.

The detection rate by PCR was even lower in our study. Applied on decontaminated tissue samples, it could detect BTB in 27.3% of suspected animals. In Brazil, 18% of cattle showing gross lesions yielded a positive PCR (Pires de Araújo et al., 2005). The *Se* of nucleic acid amplification can be influenced by different factors, such as insufficient quantity of bacilli or the presence of inhibitors hampering the polymerase (Mangiapan et al., 1996; Pires de Araújo et al., 2005; Cardoso et al., 2009). In Brazil, comparison between different concentrations and volumes of DNA samples showed that the percentage of PCR-positive lymph nodes increased from 39.4% to 54.5% after re-analysis of the initially negative samples using a 1:2 dilution of the suspension and 2,5 μl instead of 1 μl (Cardoso et al., 2009). In our study, we used 10 μl of undiluted DNA extract for the PCR, implicating that the sensitivity of our PCR might increase by the use of diluted DNA extracts, thus minimizing the effect of possible inhibitors.

We observed a good agreement between culture and PCR ($\kappa = 0.61$), implicating that a high number of samples yielded a positive result by both tests, and confirming that culture and PCR are the most important diagnostic tools for the diagnosis of BTB in dairy areas (Cardoso et al., 2009).

Histopathology showed BTB compatible images in 27.3% of the cattle with macroscopic lesions. Demelash et al. (2009) showed that the *Se* of histopathology diagnosis can be affected by the technique used and the number of slides examined. In our study, two slides per specimen were examined, i.e., after HE and after ZN staining. On the other

hand, only 5 of the 9 animals identified by histopathology were positive by all laboratory tests.

The diagnostic accuracy of a test is primarily defined by its *Se* and *Sp*. However, these parameters can be influenced by minor changes occurring when processing samples. In this study, culture was considered the gold standard method to estimate the *Se* and *Sp* of the remaining diagnostic tests. It is important to consider that all samples were paucibacillary and pre-analytical sample conditions were suboptimal for culture because of the two freezing-thawing cycles. Therefore, the observed sensitivities of PCR (56.5%), microscopy and histopathology (43.5% each) could be overestimated. Microscopy is known to have a low *Se*, as it requires 5000 to 10,000 bacilli/mL of sample to yield a positive result. The *Se* of PCR and histopathology could be influenced by factors described above.

In routine conditions, it is not feasible to apply all these laboratory tests for all samples, given the high cost and labour required. Therefore, the best strategy should be chosen to confirm the suspected cases when implementing a BTB control program in Ecuador. The results obtained in this study suggest that PCR is a good alternative for culture, yielding results in a much shorter time. However, as it counts for culture, PCR needs to be performed by trained personal in an appropriate, quality-ensured laboratory. On the other hand, microscopy is rapid, easy, and cheap, and it can be implemented in resource-poor settings (Hirao et al., 2007). Because of its low sensitivity, all smear-negative samples should be retested by culture and/or PCR. A possible strategy to perform post-mortem diagnosis for BTB could be as follows: veterinary inspection, sampling of tissues showing gross visible lesions and lungs, smear microscopy to detect AFB, and PCR on all smear-negative samples.

Our second objective was to study and characterize the distribution of lesions observed in the organs from slaughtered cattle as a surrogate marker for the possible route of transmission and stage of disease (Phillips et al., 2003). During our survey, macroscopic lesions were found in mediastinal (51.32%), tracheobronchial (23.68%), hepatic (11.84%) and retropharyngeal lymph nodes (9.21%), and in other sites (3.95%). Mild lesions in lungs were found in only one slaughtered bovine. Similar observations were published in the United States and Iran, where no gross lesions suggestive of BTB were observed in lungs from dairy cattle, but frequently in lymph nodes of the thoracic region (Whipple et al., 1996; Tadayon et al., 2008). On the contrary, affection of lungs and other organs has been reported in cattle in Africa. In Ethiopia, 84% of visible lesions were found in the lungs and thoracic lymph nodes (Teklul et al., 2004). In Mali, 79% of the animals had confirmed lesions in lungs, and the infection with *M. bovis* was highly associated with these lesions ($p < 0.001$) (Müller et al., 2008). However, in Tanzania, visible lesions were mainly found in the gastrointestinal tract (61.3%) (Cleaveland et al., 2007); these differences could be attributed to the breeds involved in the studies.

It is important to also consider that different *M. bovis* genotypes may differ in pathogenesis and thereby affect the development of lesions (Goodchild et al., 2003; Ameni et al., 2007; Oloya et al., 2007). In our study all animals with macroscopic lesions were Holstein Friesian and infected by the same strain (SB0980; Proaño-Pérez et al., in preparation). The observed inter-animal heterogeneity of macroscopic and microscopic lesions therefore most likely represents the individual diversity of pathological evolution of BTB (Meikle et al., 2007).

The macroscopic lesions found in dairy cattle slaughtered during this survey suggest that the most important route of transmission among the cattle studied was through respiratory tract.

5. Conclusion

In conclusion, our data demonstrate that veterinary inspection in combination with laboratory-based analyses is useful to document the presence of BTB, and they urge for the implementation of a national BTB control program. It should combine *in vivo* skin testing before slaughter to identify early infections with *M. bovis*, with extended post-mortem inspection including laboratory-based diagnosis. These activities should be performed by qualified veterinarians and technicians, following strict safety procedures to avoid human infections. Veterinary inspection needs to be implemented as a routine procedure in all abattoirs located in areas with a high cattle density. However, this can only be achieved if BTB is declared an important disease for animal and human health in Ecuador, and the required financial support is provided to support inspections, testing and compensation for losses.

Acknowledgements

The authors gratefully acknowledge the financial support from 'Commission Universitaire pour le Développement' (CUD), University of Liege – Project PIC. We also thank Dr. Antonio Viteri and all slaughterhouse workers in the Mejia canton for their collaboration during our survey. Finally, we thank the important contribution of the Reviewers and Associate Editor from this Journal.

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