Bacteriological diagnosis of bovine tuberculosis in bovines positive to the tuberculin test

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ABSTRACT

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB), considered a zoonotic disease. In our country, the diagnosis is based on the application of the tuberculin skin test (TST). Conventional culture, from various biological samples, can complement the TST test results. Regarding this, it is possible to perform sampling and subsequent analysis of nasal discharge, milk and tissues. The latter allows the observation of macroscopic lesions, the anatomical location in addition to collect biological samples to be analyzed by bacteriology and histopathology. The aim of this study was to evaluate the presence of M. bovis in samples of nasal secretions, milk and tissues in cattle PAC-positive and characterize gross and microscopic lesions compatible with BTB. A total of 744 samples belonging to 572 cattle from dairy herds in the provinces of Córdoba, Santa Fe and Buenos Aires, were processed. All of them were, during this study, in stage of control of BTB showing a low prevalence (between 0.5 to 4%). About 12.4% (71/572) M. bovis isolations from cattle examined was obtained. These isolations were from tissue samples 36.5% (38/104), followed by milk 10.7% (23/214) and nasal secretions 3.9% (10/254). Bacteriology from milk samples and nasal secretions were of limited effectiveness. On the other hand, tissue analysis was more useful as complementary diagnostic, allowing confirmation of suspected cases of BTB.

Keywords: Mycobacterium bovis, bacteriology, tissues, cattle.

INTRODUCTION

Mycobacterium bovis is the etiological agent of the bovine tuberculosis (BTB), an infectious zoonotic disease that causes serious productive, economic and public health problems (Grange et al., 2001). It is estimated that 500 million cattle are infected with Mycobacterium bovis in the world, causing an economic loss estimated at USD 3 billion (Hewinson et al., 2001). A study carried out in Ireland (Boland et al., 2010) attributed a 10% decrease in annual milk production in cattle positive to intradermal reaction, when compared to those not infected.

BTB’s control and eradication program in Argentina is based on the identification and elimination of infected animals through the intradermal application of the tuberculin skin test (TST) (Secretariat of Agriculture, 2012). In addition, monitoring and epidemiological surveillance is established in packing houses, where animals are inspected in order to find lesions compatible with BTB (LCT).

The TST plays a key role in diagnosis, it is the reference test worldwide, widely used as a screening test for the in vivo diagnosis of the disease. According to current regulations (Ministry of Agriculture, 2012), a dairy farm must demonstrate the absence of positive animals over 3 months, during two consecutive TSTs to be certified as “BTB Free Farm”.

Different studies attribute the TST different effectiveness values, ranging from 70% to 90% and from 75% to 99.8%...
for sensitivity and specificity, respectively (Costello et al., 1997, De Kantor et al., 1984 Francis, 1978).

These values are related to the prevalence in each farm property and can directly influence the predictive value of the diagnostic test.

The possibility of a false positive result in an infected animal may be mainly due to inespecific reactions caused by other pathogenic mycobacteria (M. avium, M. avium subsp. Paratuberculosis) and environmental factors (De la Rua-Domenech et al., 2002).

The presence of LCB can be a good diagnostic complement, while the use of diagnostic methods such as bacteriology and histopathology can generate additional information to the TST in order to understand the health status of animals suspected of BTB. Several studies demonstrated the diagnostic use of bacteriology from nasal secretions (De Souza Figueiredo et al., 2010; Neill et al., 1998), milk (Pérez et al., 2002; Zarden et al., 2013) and tissues (Liébana et al., 1995, Whipple et al., 1996), which is why it was considered as a reference method to confirm the diagnosis of BTB.

The aim of this work was to isolate and identify M. bovis from nasal secretion, milk and tissues from cattle positive to the TST, and to describe the presentation of lesions compatible with BTB.

MATERIALS AND METHODS

A cross-sectional observational study was carried out between January 2005 and January 2012. A total of 572 Holando-Argentino cattle were selected from 24 dairy herds in the provinces of Córdoba, Santa Fe and Buenos Aires. These rodeos presented an apparent prevalence that ranged between 0.5 and 4%. Animals were in sanitation stage during this work. All the bovines incorporated to this study were TST positive, and those test were performed according to the current regulations (Secretariat of Agriculture 1999 and 2012). The collected biological samples were the following:

Ante mortem

Nasal discharge

Samples of nasal secretion were collected using sterile swabs, after securing the animal in a trap. Two samples were obtained per animal by introducing the swab in each ollar at least 3 times with ascending and descending movements. The samples were conserved at 4 ºC and then decontaminated using the Petroff method (Jorge et al., 2005). They were later streaked in duplicate in Stonebrink and Löwenstein Jensen media and incubated at 37 ºC for two months. The obtained mycobacterial developments were observed under a microscope (smear microscopy), after Ziehl Neelsen (ZN) staining in order to identify AFB.

Milk

The mammary gland was sanitized and disinfected according to the procedures foreseen in the milking routine described in the “Guide to Good Dairy Farming Practice” (FAO, 2012). After removing the first secretions from each quarter of the udder, samples including the four mammary quarters of each animal (50 ml each) were collected in duplicate. The samples were refrigerated at 4 ºC and then processed with the same methodology described for the nasal secretion sample.

Post mortem

Tissues

The TST-positive cattle were subjected to necropsy or detailed inspection in the packing house. We noted and described the presence of lesions compatible with BTB. Subsequently, samples were collected in duplicate, whether or not they presented LCB. One was stored at 4 ºC and the other was buffered in 10% formaldehyde buffer, for bacteriological and histopathological studies, respectively.

Bacteriology

The collected samples were grouped into three sets, named as follows: head (HEA), formed by retropharyngeal and submandibular lymph nodes (LN); Respiratory (RES): tracheobronchial, mediastinal and lung nodes; and finally digestive (DIG): mesenteric, liver and liver lymph nodes. The tissues were refrigerated and then mechanically homogenized for 3 min. (Masticator Basic IUL Instruments model No. 470) to then proceed with the decontamination (Petroff method) and streaked in the same way as described for the previous samples. Cattle that presented more than one AFB isolate in different organs were considered as the only result.

Pathological characterization

The previously described organs were reviewed, considering LCB any area presenting focal or multifocal white-yellowish necrosis, with or without mineralization, encapsulated or not by connective tissue (Neill et al., 1988). The samples fixed in 10% formaldehyde solution were processed for microscopic study following routine procedures, until obtaining 3 μm thick sections, which were stained with hematoxylin and eosin. We considered a sample compatible with M. bovis infection all that epithelioid macrophage accumulation accompanied by lymphocytes that may or may not have a center of necrosis and mineralization, Langhans type giant cells, and connective tissue limiting the lesion. In those organs with LCB, the ZN staining was used on tissue sections in order to identify AFB.

Identification of mycobacteria

The growths obtained in culture media in which AFB were detected were identified using the polymerase chain reaction (PCR), and using as target the insertion sequence (IS) 6110 (characteristic of the Mycobacterium tuberculosis complex) (Hermans et al., 1990). The positive isolates were genotyped by Spoligotyping, as described by Kamerbeek et al. (1997).
The Mycobacterium tuberculosis complex isolates were considered positive when they were PCR (IS) 6110 positive, whereas M. bovis was considered when they were PCR (IS) 6110 positive followed by Spoligotyping. The negative isolations to PCR were analyzed using PCR-hsp65 with the aim of identifying species included in the genus Mycobacterium, according to the description of Telenti et al. (1993).

The DNA from colonies that grew in the culture media was extracted by thermal lysis. Samples were placed in 250 μL of sterile bidistilled water and incubated at 95 ºC for 45 min.

In both cases the amplification conditions were performed according to the description of Zumárraga et al. (2005).

RESULTS

The obtained results are summarized in Table 1. The findings obtained from each biological sample analyzed are described below.

Isolations in nasal discharges

We analyzed 254 samples from the selected animals. 11.4% (29/254) of them developed AFB. Of these isolates, 3.9% (10/29) were identified as M. bovis by Spoligotyping. The remaining 19 isolates were PCR-hsp65 positive, and thus we considered them as Mycobacterium spp.

Isolations in milk

We analyzed 214 samples from the selected animals, and we obtained isolated in 17% (37/214) of the samples. Of them, 62% (23/37) were identified as M. bovis. The remaining 38% (14/37) corresponded to species of the genus Mycobacterium spp. after having tested positive for hsp65-PCR.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Type of sample</th>
<th>Number of analyzed animals</th>
<th>Isolates M. bovis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ante mortem</td>
<td>Nasal discharge</td>
<td>254</td>
<td>10 (3,9)</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>214</td>
<td>23 (10,7)</td>
</tr>
<tr>
<td>Post mortem</td>
<td>Tissues</td>
<td>104</td>
<td>38 (36,5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>572</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 1. Summary of the isolates obtained from the different analyzed samples.

Bacteriology

We analyzed 276 samples of 104 positive TST cattle. Samples were obtained from necropsies or packing houses. 36.5% (38/104) of isolates belonged to the Mycobacterium tuberculosis complex, as shown in Figure 1. In 76% (29/38) of them the presence of M. bovis was confirmed, and we were not able to confirm it in the remaining 24% (9/38). 42% (16/38) of these isolates were obtained from RES samples, 21% (8/38) from the HEA samples, and 18.5% (7/38) from the DIG samples. We obtained 18.5% (7/38) isolates in two different tissues from the same animal: 4 of them were in HEA and RES, 2 in HEA and DIG, and the remaining in RES and DIG. It was not possible to obtain, from any animal, isolates from the three groups of sampled tissues.

Of the isolates belonging to the Mycobacterium tuberculosis complex, 79% (30/38) came from cattle with LCB, while 8% (3/38) corresponded to isolates obtained from cattle that did not have LCB.

The remaining 13% (5/38) was not inspected post mortem.

DESCRIPTION AND CONCLUSIONS

The results obtained from nasal secretion suggest that the isolation of the agent in that sample is of low sensitivity.
to detect M. bovis in TST-positive cattle. In this work we obtained values lower than those found in other studies, in which the proportions of isolates of M. bovis in naturally infected cattle were higher than the one described here (3.9%), and in those cases ranged between 6.7% and 20% of the studied cattle, respectively (Neil et al., 1988; Rempt, 1954).

These differences could be attributed to factors such as the intermittent elimination of mycobacteria (De Souza Fregeirodo et al., 2010), the presence of nonviable mycobacteria in the collected material, or to the fact that the used decontamination methodology affects the survival of mycobacteria (Cousins et al., 1992; Stewart et al., 2013).

Another factor to consider is the possible presence of false positive TST bovines within the study group, in addition to the fact that not all of them could be inspected post mortem. The detection limit of the bacteriological test must also be considered, in which values higher than 10 or 100 viable microorganisms are required to obtain a positive result. This amount of microorganisms is generally associated with an advanced stage of the disease, so in certain stages of this disease they would not be detectable (Barry et al., 1993; Bates et al., 1986).

Accordingly, an increase in the elimination levels by this route was described during the first 4 weeks post-infection in experimental infections, and these levels subsequently decreased (Kao et al., 2007). It was also described that during the infection there may be prolonged periods in which the microorganism can not be isolated from nasal secretions(Kao et al., 2007; McCorry et al., 2005; Neill et al., 1998).

In the case of milk samples, the isolations obtained in this work (10.7%) were higher to results previously described by Pérez et al. (2002), who detected M. bovis in 0.7% of the samples of TST-positive cattle using a similar methodology. Also, Romero et al. (1999) did not obtain isolates after analyzing 200 milk samples from TST-positive cattle. These differences could be related to aspects similar to those previously mentioned for nasal discharge.

From the point of view of public health, these findings reinforce the need to ensure the thermal treatment of milk intended for consumption (pasteurization). Also, from the sanitary-productive point of view, it should be considered that those bovine that eliminate bacteria using this via can facilitate the transmission of the disease to their young or to a group of calves that under artificial breeding systems and are fed with raw milk (Evangelista et al., 1996; Garro et al., 2011; Garro et al., 2011).

The proportion of isolates obtained in tissues (36.5%) was lower than that described in Brazil (51.5%) (Cardoso et al., 2009) and in Great Britain (50%) (Liébana et al., 1995, but higher than that reported in Ethiopia (11%) (Berg et al., 2009). This difference could be related to some of the aforementioned variables, such as the type and quantity of samples collected per animal under study, the decontamination methodology applied in each case, as well as the low number of viable mycobacteria in a tissue with LCB (Araujo et al., 2005; Miller et al., 2002).

It should also be noted that the cattle analyzed came from establishments under sanitation and with low prevalence. Although the TST is considered a specific method, its positive predictive value would decrease, thus increasing the probability that some of the animals included in this study were false positives.

This aspect could directly affect the efficiency in the isolation of M. bovis from the different analyzed samples (nasal secretion, milk and tissues).

To determine whether there are or not macroscopic lesions is useful for the diagnosis in packing houses or during necropsy. According to the results obtained, the probability of finding LCB is greater when performing a meticulous post mortem review. In commercial packing houses the time devoted to the inspection and sampling is limited and the presence of a single lesion may not be evident.

The isolation ratio of M. bovis from LCB was 61%. Similar values to those presented in this work were obtained in a study conducted between 4 laboratories in Argentina, aimed at determining the percentage recovery by culture of M. bovis from samples of tissues with LCB (Garbaccio et al., 2016).

The isolates obtained from tissue samples (36.5%) were higher than those obtained in samples of nasal secretion (3.9%) and milk (10.7%).

This difference can be attributed to the fact that in the host the microorganism lives intracellularly, and in this way if found in white tissues. For this reason, this type of sample acquires greater relevance when isolating M. bovis.

In accordance with what was described by Whipple et al. (1996), the majority of the isolations were obtained from respiratory organs (42%), followed by the samples belonging to HEA (21%), while those obtained from DIG were less frequent (18.5%). These results indicate that, like what has been described in other studies, the most important route of transmission in adult cattle is respiratory (Goodchild et al., 2001; Morris et al., 1994; O’Reilly et al., 1995; Pritchard, 1988).

Unlike what happened with the milk and nasal secretion samples, no isolations of Mycobacterium spp. were obtained in tissue samples. These findings could be related to the fact that mycobacteria considered atypical can normally be found in the environment (Oriani et al., 2002), and thus be present in airways and mammary gland, and therefore in the sample of nasal secretions and milk, respectively.

On the one hand, most of the animals of cattle (78%) presented a single LCB lesion. This value is higher than the 66% described by Corner (1994) and Corner et al. (2012). This information would indicate the need for a thorough inspection of the tissues during the slaughter or the necropsy to avoid the possible release to contaminated material.

On the other hand, 8% of the isolates obtained from LCB-free animals during post-mortem inspection. Whipple et al. (1996) described an even higher proportion (10%) of isolates obtained from tissues that did not have LCB. Similar
results were obtained after the histopathological study since 9.3% of the cases considered positive in this trial did not present visible macroscopic lesions.

Based on what has been described in different studies (Garbaccio et al., 2010; Parra et al., 2008), the incorporation of direct tissue PCR would be complementary to the methodology we used (inspection of lesions, bacteriology and histopathology), thus improving the sensitivity as well as decreasing the work time required for bacteriology (2 days versus 60 days).

The results presented in this work indicate the importance of the bacteriological diagnosis from the different samples analyzed. The isolation of M. bovis from milk and nasal secretion would have a limited diagnostic utility, although the samples can be easily obtained from the live animal. On the contrary, the bacteriological analysis of tissues would be of greater diagnostic relevance, as was described by Mantilla et al. (2009).

Its use would allow understanding health events that require a detailed analysis, as would be the case of a BTB free farm where new reactors to the TST arise, or the case of those with a low number of reactive animals, suspected of possible false positive results to the TST. In this way, streaking followed by molecular typing allows us to resolve specific situations, and to provide complementary information about the circulating strains in a farm or region and its potential use from the epidemiological point of view.

The effective implementation of the different available diagnostic methods should be taken into account for their strategic use in accordance with the provincial or national programs for the control and eradication of bovine tuberculosis.

REFERENCES


