Tuberculosis and paratuberculosis co-infection in dairy cattle in Morocco

M. EL MRINI[1], J. BERRADA[1], F. KICHOU[1], B. ROMERO[2], A. BALSEIRO[3], M. BOUSLIKHANE[1]

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Abstract

Bovine tuberculosis (BTB) is a major endemic zoonosis in Morocco, Northern Africa. Bovine paratuberculosis (PTB) has an unknown epidemiological status in Morocco. We aimed to investigate TB/PTB co-infection in 6 dairy cattle farms (A to F) and to generate information on the possible PTB host status in this region. *Mycobacterium bovis* was isolated in 25 of 225 (11%) dairy cows. Isolates yielded 7 spoligotypes: SB 0120, SB 0121, SB 0125, SB 0265, SB 0869, SB 1167 and SB 1265. *Mycobacterium avium* subsp *paratuberculosis* (Map) was isolated in 9 (4%) of 225 dairy cows. Thus TB/PTB co-infection was diagnosed in 3 dairy cattle herds (A, B and E). Furthermore, in herd E, intra-individual TB/PTB co-infection was diagnosed in three cows. Our results confirm TB/PTB co-infection in Moroccan dairy cattle. However, our information was limited to only 225 dairy cows in the northern third of Morocco. Thus, further research is needed to assess the dairy cattle status in the whole country.

Key words: Tuberculosis, paratuberculosis, co-infection, dairy cattle, Morocco.

Co-infection tuberculose-paratuberculose chez des vaches laitières au Maroc

Résumé

La tuberculose bovine (TB) est une zoonose endémique majeure au Maroc. La paratuberculose bovine (PTB) ne présente pas de statut épidémiologique connu au Maroc. Le présent travail a porté sur l'investigation de la co-infection TB/PTB au niveau de 6 élevages de bovins laitiers (A à F) afin de générer des informations sur le possible statut de la PTB sur le terrain. *Mycobacterium bovis* a été isolé chez 25 (11%) parmi 225 vaches laitières. Les isolats de *M. bovis* appartiennent à 7 spoligotypes: SB 0120, SB 0121, SB 0125, SB 0265, SB 0869, SB 1167 et SB 1265. *Mycobacterium avium* subsp *paratuberculosis* (Map) a été isolé chez 9 (4%) parmi 225 vaches laitières. Ainsi la co-infection TB/PTB a été diagnostiquée au niveau de trois élevages de vaches laitières (A, B et E). En outre, au niveau de l'élevage E, la co-infection TB/PTB au niveau intra-individuel a été retrouvée chez trois vaches laitières. Ces résultats confirment la co-infection TB/PTB au niveau des élevages bovins laitiers au Maroc. Cependant, notre information a été limitée à 225 vaches laitières au niveau du tiers nord du Maroc. Ainsi, d'autres recherches sont nécessaires pour évaluer le statut des bovins laitiers sur l'ensemble du pays.

Mots clés : Tuberculose, paratuberculose, co-infection, bovins laitiers, Maroc.

INTRODUCTION

Animal tuberculosis (TB) is due to infection with *Mycobacterium bovis* and other closely related members of the *Mycobacterium tuberculosis* complex (MTC). TB is relevant for public health (mainly in developing countries), animal health and production, since it causes severe economic losses to the livestock industry (Gortázar *et al.*, 2015). TB is a major endemic zoonotic disease in Morocco, with 33% of dairy cattle farms infected countrywide with mycobacteria of *Mycobacterium tuberculosis* complex (MTC) (FAO, 2004). In 2017, a total of 2.640 tonnes of red meat and giblets were seized with bovine tuberculosis as the major seizure reason for meat (ONSSA, 2017).

Moroccan public animal health authority recognizes single and comparative intradermal tuberculin (IDT) tests as the official bovine TB field screening tests. Interferon-gamma (IFN-γ) assay may be authorized to maximize early infected cattle detection.

Paratuberculosis (PTB) is a chronic infectious disease caused by *Mycobacterium avium* subsp *paratuberculosis* (*Map*) and is characterized by chronic granulomatous enteritis which leads to chronic diarrhea and progressive emaciation in ruminants (González *et al.*, 2005). In Morocco, paratuberculosis in small ruminants has been diagnosed and characterized by several studies (Benazzi *et al.*, 1995; Bauerfeind *et al.*, 1996; Benazzi *et al.*, 1996). However, bovine paratuberculosis (PTB) has an unknown epidemiological status in Morocco.

PTB infection has been demonstrated to compromise the specificity of TB screening tests (Chiodini *et al.*, 1984; Monaghan *et al.*, 1994). Particularly some pathological forms of PTB which produce a strong cellular immune response (mainly multifocal and diffuse lymphocytic and intermediate lesions) could interfere in IDT (Balseiro *et al.*, 2003). In the present study, we aimed to investigate TB and PTB co-infection in dairy cattle in Morocco.

MATERIALS AND METHODS

Herds/Specimens

Between 2013 and 2016, TB/PTB suspected cases in six dairy cattle farms (A to F) were investigated at the Department of Veterinary Pathology and Public Health at Hassan II Institute of Agronomy and Veterinary Medicine in Rabat, for bacteriology analyses and histopathology. Dairy cattle farms were located respectively A in Fes, B in Sidi Slimane, C and D in Sidi Kacem, E in Benslimane and F in Larache. Tissue specimens not showing TB-like

[1] Department of Veterinary Pathology and Public Health, Hassan II Institute of Agronomy and Veterinary Medicine, Rabat, Morocco
[2] Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Madrid, Spain
[3] Centro de Biotecnología Animal-SERIDA Gijón Asturias Spain

lesions were collected from two hundred and twenty-five culled IDT-positives, predominantly adult Prim'Holstein cows, in slaughterhouses controlled by public veterinary practitioners.

Sampled tissues were divided in a first lymph node pool including retropharyngeal, prescapular and mediastinal lymph nodes (280/587, 47.7%), a second lymph node pool including mesenteric and iliac lymph nodes (196/587, 33.4%) and a third pool including ileo-caecal valves and terminal ileons (111/587, 18.9%). Culture was performed as the gold standard to determine the true infection status of the studied herds (OIE, 2014).

Bacteriology

Bacteriology was conducted on a total of 587 tissue samples from 225 dairy cows. Retropharyngeal, prescapular, mediastinal and iliac lymph nodes samples were desiccated to remove adipose tissue, and 2 g of the desiccated tissues were mixed with 2 g of sterilized sand and 10 ml of phenol red. The solution (7.5 ml) was placed in a 50 ml conic tube, 2.5 ml of NaOH 1 N was added at room temperature for 10 min, and then HCl 6 N was added for sample neutralization. As a final step, the tube was centrifuged for 25 min at 3500 rpm. The supernatant was discarded and pellet was distributed in the following culture medias Lowenstein-Jensen (LJ), LJ with glycerol (LJG) or pyruvate (LJP) and Herrold according to the availability in the laboratory. Cultures were incubated for 20 weeks at 37 °C and observed daily for growing colonies during the first week then weekly from the second week onwards (Hobby, 1962).

Ileo-caecal valves, terminal ileons and mesenteric lymph nodes were desiccated to remove adipose tissue, and 2 g of the desiccated tissues were mixed with sterilized sand and 3 ml of phenol red. The solution (2 ml) was placed in a 50 ml conic tube containing 45 ml of Benzalkonium chloride (0.1%) at room temperature for 2 min, and then the tube was centrifuged for 10 min at 1500 rpm. The supernatant was discarded and pellet was distributed on Herrold media with (3 tubes) and without (1 tube) mycobactine. Cultures were incubated for a maximum of five months at 37 °C and observed daily for growing colonies during the first week then weekly from the second week onwards (Merkal and Cullough, 1982; Merkal, 1984).

Molecular biology

Acid-fast bacilli were confirmed by Ziehl-Neelsen (ZN) staining of any compatible colonies. All the grown cultures were desactivated by adding a loopful of *Mycobacterium* sp colonies to 1 ml of sterilized distilled water contained in small sterile tubes. The samples were then inactivated for 10 minutes at 100 °C. Molecular biology investigation was performed in the Center for Veterinary Health Surveillance VISAVET at Complutense University in Madrid. *Mycobacterium* sp isolates were characterized by using Multiplex PCR (IS6110 for MTC, 16S ARNr for *Mycobacterium* genus) (Wilton and Cousins, 1992; Sevilla *et al.*, 2015) and IS 900 for *Map* (Collins *et al.*, 1989). Spoligotyping patterns (Kamerbeek *et al.*, 1997) were defined according to *Mycobacterium bovis* database.

Histopathology

Ninety-four tissues from 51 dairy cows from herd E, including ileo-caecal valves (49%) and mesenteric lymph nodes (51%), were fixed in 10% neutral-buffered-formalin and processed for histopathological examination by standard methods. Five-µm thin paraffin sections were stained with hematoxylin and eosin (H&E) and Ziehl-Neelsen stains for acid fast bacilli.

Immunohisto-chemistry

Immunohisto-chemistry (IHC) was performed in the Animal Biotechnology Center SERIDA at Gijón Asturias, in Spain. IHC was used to confirm the presence of *Mycobacterium avium* subsp *paratuberculosis* (*Map*) using the Avidin Biotin Complex (ABC) method (Vector Laboratories) following the manufacturer's instructions. Briefly, the sections were incubated with specific rabbit-antiserum at a dilution of 1:1000, prepared previously by the hyper-immunization of two rabbits with a sonic extract of a local *Map* strain (A-82) isolated from the intestinal tissues of a cow (Balseiro *et al.*, 2003). To evaluate the specificity of the anti-*Map* antibody, tissue samples from a paratuberculous cow were used. Negative controls from a healthy cow were also use in each run.

RESULTS AND DISCUSSION

Mycobacterium bovis isolates

Mycobacterium bovis was isolated in 25 dairy cows from four herds (A, B, E and F) (Table 1). M. *bovis* isolates yielded 7 spoligotypes: SB 0120, SB 0121, SB 0125, SB 0265, SB 0869, SB 1167 and SB 1265 (Table 1). The herds A, B and F were having TB eradication program for several years and observing biosecurity measures. This contest may explain the one or two *M. bovis* isolated spoligotypes. The herd E was starting TB eradication program and biosecurity approach, thus will explain the several *M. bovis* spoligotypes in this farm.

Table 1: PCR and spoligotyping results

Herd	Cattle effectives in mycobacteriology	*Mycobacterium bovis* spoligotype	*Mycobacterium avium* subsp *paratuberculosis* isolates	*G. Mycobacterium* isolates
A	70	SB 0120 (x1) SB0125 (x1)	2	2
B	49	SB 1265 (x1)	1	1
C	48	-	1	4
D	5	-	-	-
E	51	SB 0121 (x2)	5	1
		SB 0265 (x2)		
		SB 0869 (x13)		
		SB 0120 (x3)		
F	2	SB 1167 (x2)	-	-
Dairy cows total	225	25 (11%)	9 (4%)	8 (3.6 %)

M. bovis spoligotypes from cattle in this study was also reported in the paper of Yahyaoui-Azami *et al.* (2017), a part *M. bovis* spoligotype SB 1167 (herd F), reported for the first time in this study. *M. bovis* spoligotypes SB 0121 and SB 0869 from cattle in the present study are matching with *M. bovis* spoligotypes isolated from human beings in Morocco (Lahlou *et al.*, 2012; Chaoui *et al.*, 2014). The *M. bovis* spoligotype SB 1627 isolated in wild boar in Morocco (El Mrini *et al.*, 2016) was not isolated in the present work.

Map and non-tuberculous mycobacteria isolates

Map was isolated in 9 dairy cows from four herds (A, B, C and E). The 2 other farms (D and F) that were not diagnosed having PTB presented low bovine samples. The available *Map* isolation results illustrate the alarming PTB presence in the field that need to be more studied in the future.

Non-tuberculous mycobacteria were also isolated in four herds (A, B, C and E). *Map* and non-tuberculous mycobacteria presence have been demonstrated to compromise the specificity of TB screening tests (Amadori *et al.*, 2002; Hope *et al.*, 2005).

Histopathology/IHC

Histopathology and IHC proved *Map* presence in paraffin embedded tissues from three dairy cows in herd E (Figure 1).

TB/PTB co-infection

TB/PTB co-infection was diagnosed in 3 dairy cattle herds (A, B and E) providing the evidence of TB/ PTB co-infection among Moroccan dairy cattle. Furthermore, in herd E, intra-individual TB/PTB co-infection was diagnosed in three cows.

Several works worldwide were published about TB/PTB co-infection in cattle herds (Amadori *et al.*, 2002; Hope *et al.*, 2005; Lilenbaun *et al.*, 2007). TB/PTB co-infection in cattle induce important economic losses due to elevate monitoring fees and cattle culling by excess due to false positive results for IDT (Dunn *et al.*, 2005). Screening by the comparative intradermal test (IDC) can reduce the amount of false positive results to bovine tuberculin (Alvarez *et al.*, 2007).

CONCLUSION

In the present work, our information was limited to only 225 dairy cows in the northern third of Morocco. Thus, further researches are needed to assess the dairy cattle status in the whole country.

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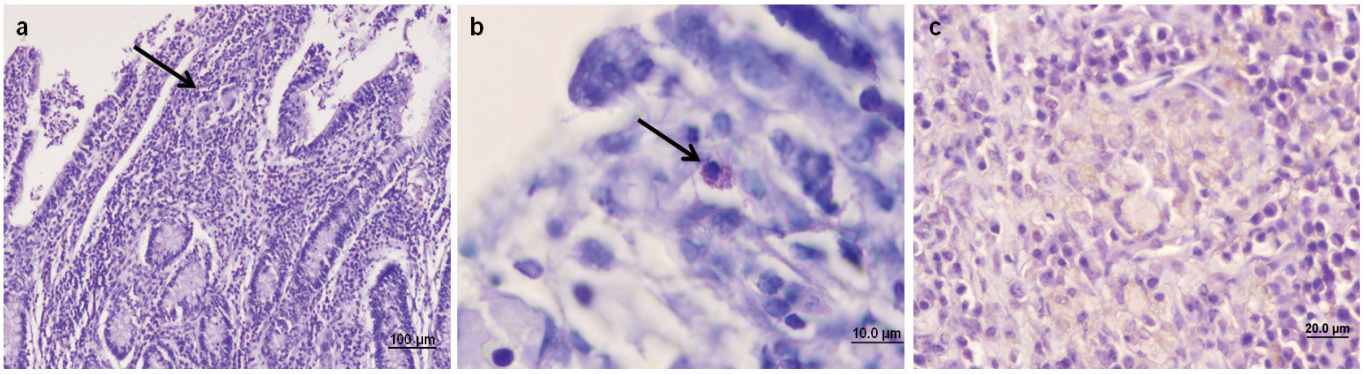

Figure 1: Histopathology and immunohisto-chemistry results illustrations

(a): ***Ileum: enteritis granulomatous diffuse with villus atrophy and Langhans multinucleated cells (arrow). Hematoxylin and eosin (H&E) stain, Bar: 100 µm;*** **(b):** ***ZN-stained acid-fast bacilli (arrow) in bovine ileum. Bar: 10 µm;*** **(c):** ***Ileum tissue section immune-stained. Positive reactions appear as distinct granular brown staining within macrophages and multinucleated cells. Bar: 20 µm***

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