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Julio Álvarez, Lucía de Juan, Javier Bezos, Beatriz Romero, Jose Luis Sáez, et al.. Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay. *Veterinary Microbiology*, 2009, 135 (3-4), pp.389. 10.1016/j.vetmic.2008.09.060 . hal-00532504

HAL Id: hal-00532504

<https://hal.science/hal-00532504>

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Accepted Manuscript

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PII: S0378-1135(08)00436-7
DOI: doi:10.1016/j.vetmic.2008.09.060
Reference: VETMIC 4202

To appear in: *VETMIC*

Received date: 22-4-2008
Revised date: 4-9-2008
Accepted date: 15-9-2008

Please cite this article as: Álvarez, J., de Juan, L., Bezos, J., Romero, B., Sáez, J.L., Marqués, S., Domínguez, C., Mínguez, O., Fernández-Mardomingo, B., Mateos, A., Domínguez, L., Aranaz, A., Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-gamma detection assay, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.060

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**Effect of paratuberculosis on the diagnosis of bovine tuberculosis in a cattle herd
with a mixed infection using interferon-gamma detection assay**

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Abstract

Interferon-gamma (IFN- γ) detection assay is being applied as an ancillary test to tuberculin tests in the diagnosis of bovine tuberculosis to detect the maximum number of infected animals. Among possible factors influencing the performance of tuberculosis-diagnostic tests, paratuberculosis, a widespread disease in Spain and other European countries, has been pointed out as a cause of false positive reactions. Still, its effect on the sensitivity of these tests in cattle has yet to be fully characterized. The impact of paratuberculosis in the apparent sensitivity of IFN- γ assay was studied in a bullfighting cattle herd with a mixed tuberculosis-paratuberculosis infection, using culture of *Mycobacterium bovis* and *Mycobacterium avium paratuberculosis* as the gold standard to determine the infection status of every animal. A total of 218 animals were slaughtered and sampled for bacteriology after blood sampling. IFN- γ assay showed a lower apparent sensitivity in animals with a mixed infection (50%) compared to all animals suffering tuberculosis (78.3%). This finding indicates that the presence of paratuberculosis in tuberculosis-infected herds could imply a serious impairment in the sensitivity of IFN- γ detection test.

Keywords: tuberculosis; paratuberculosis; cattle; IFN- γ ; diagnosis.

1. Introduction

Bovine tuberculosis is a zoonotic disease affecting cattle worldwide, causing economic losses due to eradication programs and trade limitations (Pollock and Neill, 2002). In spite of great efforts the Officially Tuberculosis Free status has not yet been achieved in some countries throughout Europe. This lack of success has been attributed, among other causes, to the insufficient sensitivity of the diagnostic tests under certain field conditions (Monaghan et al., 1994; Morrison et al., 2000).

In Spain prevalence of bovine tuberculosis has followed a decreasing trend from 11.14% (herd level) and 4.2% (individual level) in 1986 (when the national eradication program was implemented) to 1.76% and 0.42% in 2006 (Anon., 2008). At the moment, detection and removal of the maximum number of *M. bovis* infected animals is the crucial step. Therefore, the increase of the sensitivity of diagnostic tests is a key objective. For this reason the interferon- γ (IFN- γ) detection assay was introduced in the EU legislation (Council Directive 64/432/EEC, amended by (EC) No. 1226/2002 of 8 July 2002, Anon. 2002) as an ancillary test and adopted by the Spanish program (Anon., 2008). The conditions for the optimal use of this technique, already demonstrated in several countries (Wood et al., 1990; Gormley et al., 2006), must be determined for the different epidemiological situations in Spain.

To our knowledge the sensitivity of the IFN- γ test in cattle naturally co-infected by *M. bovis* and *Mycobacterium avium paratuberculosis* (*Map*) has not been studied under field conditions. A decreased sensitivity was reported by Amadori et al. (2002) and Hope et al. (2005) in cattle previously infected with *M. avium* under experimental conditions, but this might not reflect the field situation of a naturally mixed infection with a *M. tuberculosis* complex member and *Map*. Aranaz et al. found a decreased sensitivity in tuberculosis diagnostic tests in a cattle herd with a dual infection, but the

59 number of animals with positive culture was low, and selection of slaughtered animals
60 could have produced a biased higher sensitivity (Aranaz et al., 2006).

61 The current study was designed to determine the possible effect of natural co-
62 infection by *M. bovis* and *Map* on the performance of IFN- γ detection assay for the
63 diagnosis of tuberculosis in a bullfighting cattle herd.

2. Materials and methods

2.1. Herd and design of the study

The study was performed in a bullfighting cattle herd with a natural mixed tuberculosis-paratuberculosis infection. The whole herd (n=680) was subjected to analysis by single intradermal comparative cervical tuberculin (SICCT) test, IFN- γ detection assay and an ELISA for the detection of antibodies against *Map*. All animals sent to the slaughterhouse (n=218) because of positive results in any test or for zootechnical reasons were sampled to perform bacteriology.

2.2. Diagnostic methods

2.2.1. SICCT test

The SICCT test was conducted by the Official Veterinary Services according to European and national legislation (EU Council Directive 64/432/CEE and RD 2611/1996) after blood sampling for IFN- γ detection using bovine and avian PPDs (CZ Veterinaria, Porriño, Spain).

2.2.2. IFN- γ detection test

Heparinized blood samples were collected from every animal and delivered to the laboratory within eight hours of collection at room temperature. Stimulation with avian and bovine PPDs was carried out as previously described (Wood et al., 1990; Liebana et al., 1998), and plasma samples were analysed in duplicate using a sandwich EIA for detection of bovine IFN- γ (Bovigam™ Bovine Gamma Interferon Test, Prionics, Schlieren, Switzerland). Results were interpreted as previously described (Aranaz et al., 2006). Animals were considered positive if the mean optical density (OD) of a sample stimulated with bovine PPD minus the mean OD of nil antigen was greater than 0.05 and greater than the same value of the sample stimulated with avian PPD (interpretation prescribed in the Spanish eradication program).

2.2.3. Serology of paratuberculosis

Plasma samples were tested in duplicate using the absorbed ELISA kit ParachekTM (Prionics, Schlieren, Switzerland) according to the manufacturer's instructions. Animals were considered positive when its mean OD minus the plate negative control was greater than 0.100 as recommended by the manufacturer.

2.3. Bacteriology

2.3.1. Tissue collection

Animals were slaughtered at the abattoir, and samples were taken at the post-mortem inspection for culture of *M. bovis* (retropharyngeal, mediastinal and bronchial lymph nodes, and lung) and *Map* (mesenteric and ileocecal lymph nodes and ileocecal valve). Samples were stored at -20°C until further analysis.

2.3.2. *M. bovis* culture and characterization

Tuberculosis compatible lesions were selected when present; otherwise, a pool of tissues was used. Culture of samples was performed after decontamination with 0.37% hexadecylpyridinium chloride (Corner and Trajstman, 1988) as described elsewhere (Liebana et al., 1998). All isolates were identified by PCR (Wilton and Cousins, 1992) and characterised using spoligotyping (Kamerbeek et al., 1997).

2.3.3. *Map* culture and characterization

Tissue samples of each animal were pooled and processed as described before (Greig et al., 1997) and inoculated onto four selective culture media (de Juan et al., 2006). Isolates were identified as *Map* based on their mycobactin- dependency and presence of specific elements IS900 and f57 (Coetsier et al., 2000). The type of strain was determined using the PCRs described by Collins et al. (2002) and by Castellanos et al. (2007).

2.4. Evaluation of diagnostic test

The infection status of each animal was determined using their culture results as the gold standard. Results from animals with a mixed infection (both *M. bovis* and *Map* were recovered from their tissue samples) were then compared with all animals infected by *M. bovis* to evaluate the apparent sensitivity of the IFN- γ assay, and with all animals infected by *Map* for the assessment of the apparent sensitivity of the Parachek ELISA.

Statistical analysis was performed by means of the software PEPI 4.0 (Computer Program for Epidemiologist), J.H. Abramson and P.M. Gahlinger.

3. Results

3.1. Bacteriology results

A total of 218 animals were slaughtered and sampled for bacteriology during the study. Ninety-eight were culled based on their positive results in the diagnostic tests for tuberculosis: six animals were positive to the SICCT test, 81 to the IFN- γ assay and 11 to both techniques. Eighty-two other animals were slaughtered as they had positive values in the serology of paratuberculosis. The remaining 38 animals were slaughtered for zootechnical reasons (inadequate temper, poor reproductive rate or lameness). *M. bovis* and *Map* were isolated from 46 and 111 animals respectively (Table 1). Two of the animals negative to all tests showed lesions compatible with tuberculosis at the abattoir inspection, and both had subsequently *M. bovis* and *Map* positive-culture.

The 46 *M. bovis* isolates belonged to six spoligotypes, from which two were much more prevalent: SB0298 (n=29), SB0339 (n=12), SB0121 (n=2), and SB0120, SB1313 and SB1321 (n=1). Regarding the *Map* isolates, 80 (72.1%) belonged to the type II and 31 (27.9%) were type III.

3.2. Impact of infection status in tuberculosis diagnosis

Animals were classified based on their culture results according to the criteria detailed above. Proportion of positive animals and apparent sensitivity were determined and compared in each group. Compulsory skin test was performed according to the eradication program; however the results are not analyzed in detail in this study because the dangerous handling of this bullfighting herd may have impaired the carrying out of the test, therefore making difficult the interpretation of results.

Regarding IFN- γ assay, a low apparent sensitivity of 78.3% (36 out of the 46 animals with *M. bovis*-positive culture) was observed, though a detailed analysis of the results revealed it could be mostly attributed to the animals with a mixed infection

(Table 2). In fact, among these animals only eight of the 16 (50%) were positive reactors in the IFN- γ assay (Table 2), although when their avian PPD readings were ignored the number of detected animals raised to 11 (68.8%) (Table 3). All of the false negative reactors to the IFN- γ test in this group (n=8) were also negative in the SICCT test. Remarkably, six out of these eight negative animals were positive in the Parachek ELISA, while most IFN- γ reactors (six out of eight) showed negative results in that ELISA (Table 3). None of these IFN- γ negative animals presented disseminated tuberculous lesions which could indicate anergy due to advanced progression of infection.

3.3. Impact of infectious status in paratuberculosis diagnosis.

The ELISA for detection of *Map* antibodies detected a lower proportion of *Map* infected animals in the group with a mixed infection (eight out of 16, 50%) compared to all *Map* infected animals (81 out of 111, 73%) (Table 2).

No association was observed between the type of *Map* strain infecting an animal and the probability of being detected by any method.

4. Discussion

In spite of investing great efforts in the fight against tuberculosis, achievement of complete eradication has been inconsistent in certain regions or countries (Neill and Pollock, 2000). Wildlife reservoirs (Corner, 2006) and dissemination due to movement of infected animals (Johnston et al., 2005; Collins, 2006) have been pointed out as possible causes of this failure. Other major factors hampering eradication programs are limitations of routine diagnostic tests (mainly skin tests), in particular concerning their sensitivity (Monaghan et al., 1994; Morrison et al., 2000). Diagnostic accuracy is a key issue in the test-and-slaughter programs applied in Spain and many other countries, especially at the present low-prevalence stage, when detection of all infected animals is crucial (Pollock et al., 2001).

In the present study the proportion of *M. bovis* infected animals detected by the IFN- γ assay seems to be greatly affected by the presence of a concomitant *Map* infection; the apparent sensitivity of the test in this herd was 78.3%, but in the group of animals with a mixed infection it dropped to 50%. The impact of paratuberculosis compromising the specificity of tuberculosis diagnostic tests has been demonstrated (Cagiola et al., 2004; Dunn et al., 2005); however, its effect on the sensitivity in cattle had not been determined in natural conditions. Nevertheless, paratuberculosis could be of great importance, as it is widespread in certain areas of the European Union (Nielsen and Toft, 2007) and other parts of the world (Kennedy and Benedictus, 2001). A statistically significant relationship (C.I. 95%) was found between the recovery of *Map* from tissue samples and response to the IFN- γ test in *M. bovis* infected animals. While 93.3% (28 out of 30) of animals with *Map* negative culture results did respond to the assay, only 50% of cattle yielding *Map* by culture was detected. Furthermore, among the ten false negative reactors found in the whole herd, eight of them (80%) were

suffering a concomitant *Map* infection.

These results corroborate the findings of a previous report in a goat flock (Alvarez et al., 2007), where paratuberculosis seemed to have a major effect on the sensitivity of tuberculosis diagnostic tests, especially on skin test, as the IFN- γ assay was less affected. On the other hand, in the present study the ability of IFN- γ test to detect tuberculosis-infected animals is more influenced by paratuberculosis; these differences could be due to the host species or the strains present in each herd.

In the group of cattle with dual infection, 50% (8/16) of the animals were false negative reactors in the IFN- γ assay, although this apparent sensitivity increases to 68.8% if interpretation is performed overlooking values obtained with avian PPD (Table 3). Further studies would be needed in order to disclose the advisability of adapting interpretation criteria of the IFN- γ assay under these circumstances.

Interestingly, 75% of these dually infected animals with no response in the IFN- γ assay were positive in the serology of *Map*, in opposition to what happened to most animals positive in the IFN- γ test in the same group (that were seronegative for *Map*) (Table 3). The negative association between results in IFN- γ assay and serology of *Map* in animals with a mixed infection was also observed in the previous report in goats (Alvarez et al., 2007) as from eight false negative reactors to the IFN- γ assay found in that study, seven (87.5%) had high titers of antibodies against *Map* (unpublished data).

Regarding the ELISA for detection of antibodies against *Map*, it is remarkable that a lower apparent sensitivity was observed in the group of animals with a mixed infection compared to the whole group of animals infected with *Map*. However, this fact would have no consequences under field conditions because animals would be culled due to positive response to the tuberculosis diagnostic tests.

In conclusion, our results would suggest that both *M. bovis* and *Map* influence

the overall immune response developed in the dually-infected animals, and may compromise the reliability of diagnostic tests that are based in the measurement of specific response generated against each pathogen. Differences in the proportion of *M. bovis*-infected animals reacting to the IFN- γ assay were observed depending on their paratuberculosis status: animals with dual infection were more likely to produce false negative reactions to this test. This fact highlights the importance of ascertaining the paratuberculosis status of a herd when implementing a strategic use for the IFN- γ assay, not only because its effect on the specificity but also for its impact on the sensitivity. More studies should be performed with larger numbers of animals representing the actual bovine population in order to confirm this finding.

Acknowledgements

This research was funded by project AGL2006-06206 of the Spanish Ministry of Science and Technology, ParaTBTools (STREP 23106) of the European Union, and the Spanish Ministry of Agriculture, Food and Fisheries. J. Álvarez was the recipient of a predoctoral grant assigned by the Spanish Ministry of Education and Culture. The group is a partner of the coordination action “Veterinary European Network on Mycobacteria (VENoMYC)” funded by the European Union.

We are grateful to F. Lozano and N. Moya for technical help; to the Official Veterinary Services for the IDTB testing and collection of blood samples; and to C. García, V. Bielsa, and A. Zabaleta [abattoir staff of Leganes (Madrid)] for assistance in collection of tissue samples. We would like to thank C. Escribano, L. Carbajo, and J. L. Paramio (Dirección General de Ganadería, Spanish Ministry of Agriculture, Fisheries and Food) for their continuous encouragement. The willingness and cooperation of landowner is greatly appreciated. We acknowledge Matthew Gilmour and Devin J. Morey for careful revision of the manuscript.

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326

Table 1.

Mycobacterium bovis and *Mycobacterium avium* subsp. *paratuberculosis* culture

results of slaughtered animals (n=218)

Culture results	<i>Map</i> +	<i>Map</i> -	Total
<i>M. bovis</i> +	16	30	46
<i>M. bovis</i> -	95	77	172
Total	111	107	218

327

Table 2.

Apparent sensitivities with confidence intervals (95%) of the IFN- γ detection test and the ParachekTM ELISA in the groups formed based on culture results

Diagnostic test	Tbc+ animals (n=46) ^a	Ptb+ animals (n=111) ^b	Tbc+/Ptb+ (n=16) ^c
IFN- γ detection test	78.3 (63.6-89)	-	50 (24.6-75.3)
Parachek ELISA	-	73 (63.7-81)	50 (24.6-75.3)

328 ^a Animals with *M. bovis* positive isolation (regardless their *Map* culture results).

329 ^b Animals with *Map* infection (regardless their *M. bovis* culture results).

330 ^c Animals with positive cultures of both *M. bovis* and *Map*.

331

Table 3.

Results of IFN- γ assay and Parachek ELISA in animals suffering a tuberculosis-paratuberculosis dual infection (n=16)

Animal	Bovine IFN- γ^a	Avian IFN- γ^b	Parachek ELISA ^c
1	0.635	0.132	-0.012
2	0.529	0.080	0.008
3	0.314	0.263	-0.025
4	0.212	0.069	-0.025
5	0.161	0.044	0.384
6	0.157	0.036	-0.020
7	0.115	0.036	-0.026
8	0.051	0.016	0.126
9	0.035	0.077	1.048
10	0.136	0.139	0.883
11	0.239	0.888	0.400
12	0.070	0.141	0.242
13	0.007	0.005	0.213
14	0.007	0.017	0.210
15	0.018	0.024	0.013
16	0.017	0.002	-0.007

332 Optical densities above the cut-off point (positive animals) are presented in bold letters.

333 ^a Results after the in-vitro stimulation with bovine PPD minus the value obtained after
334 stimulation with PBS.

335 ^b Results after the in-vitro stimulation with avian PPD minus the value obtained after
336 stimulation with PBS.

337 ^c Results in the ELISA for detection of *Map* antibodies.