Original article

Assessment of diagnostic tools for eradication of bovine tuberculosis in cattle co-infected with *Mycobacterium bovis* and *M. avium* subsp. *paratuberculosis*

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Abstract – The intradermal tuberculin (IDTB) test and the interferon-gamma (IFN-γ) assay are used worldwide for detection of bovine tuberculosis in cattle, but little is known about the effect of co-infecting agents on the performance of these diagnostic tests. This report describes a field trial conducted in a cattle herd with dual infection (bovine tuberculosis and paratuberculosis) during 3.5 years. It has been based on a strategic approach encompassing serial parallel testing (comparative IDTB test, the IFN-γ assay and serology of paratuberculosis) that was repeated 8 times over the period, and segregation of animals into two herds. The IDTB test detected 65.2% and the IFN-γ test detected 69.6% of the *Mycobacterium bovis* culture-positive cattle. However, the IDTB test performed better during the first part of the trial, while the IFN-γ test was the only method that detected infected animals during the following three samplings. The number of false positive reactors with the IDTB and/or the IFN-γ tests was remarkably high compared to other reports, and could be caused by cross-reactivity with *M. avium* subsp. *paratuberculosis*. Also, the *M. bovis* isolates from cattle and wildlife from the same property were characterised using molecular techniques to disclose an epidemiological link. The IDTB test may not be appropriate to eradicate bovine tuberculosis in herds with dual mycobacterial infections. This report highlights the need to use several diagnostic techniques for the accurate detection of *M. bovis* infected animals in these herds.

tuberculosis / paratuberculosis / IDTB / IFN-γ / eradication

1. INTRODUCTION

Eradication of bovine tuberculosis, zoonoses caused by *Mycobacterium bovis,* relies on the detection of infected animals and subsequent slaughter of reactors. The intradermal tuberculin (IDTB) test has been used for routine field detection of infected animals since nearly a century ago [19], and it is the official test in most countries.

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A review estimates the sensitivity values of the tuberculin test (at standard interpretation) to be 90% [22]. Failure to diagnose animals infected by *M. bovis* results in the persistence of infection and may contribute to cattle to cattle spread. In Spain, the programme for the eradication of bovine tuberculosis is based on the use of the IDTB test according to Council Directive 64/432/EEC[1] and removal of reactors. In 2004, this test was positive in 0.40% of animals (1.80% of herds). Despite the progress achieved over recent years, bovine tuberculosis still poses a serious problem in specific regions of the country, such as areas of extensive management properties and coexistence of abundant wildlife, thus, the use of additional approved techniques would be desirable to speed up the eradication.

In order to enhance sensitivity, specificity, and to reduce handling events, Wood et al. described the gamma-interferon (IFN-γ) test [43]. This test is an in vitro immunoassay based on the specific release of IFN-γ as the indicator of a response to the *M. bovis* antigen (bovine purified protein derivative, bovine PPD). IFN-γ can be detected using an enzyme immunoassay [29] which is commercially available.

Several field trials have compared the performance of IDTB and IFN-γ tests during the last decade. In most of them, the sensitivity of the IFN-γ test was higher compared to the tuberculin tests [13, 18, 24, 28, 32, 44, 45]. The specificity of the IFN-γ assay was 90.6 to 98.6% [3, 20, 44]. However, some factors have been suggested to affect the specificity of the IDTB test and the IFN-γ assay, and the most frequent could be the presence of other mycobacterial infections such as paratuberculosis. Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subspecies *paratuberculosis,* is a chronic granulomatous enteritis of bovids. Paratuberculosis

has been recognised to be present worldwide, though with important differences in prevalence (reviewed in [16]).

It has been reported that vaccination against paratuberculosis of goats with an inactivated vaccine[2], and of cattle with a live modified vaccine [17] or an inactivated vaccine [23] could compromise the specificity of both the IFN-γ and SIDT tests. It is suspected that paratuberculosis itself may have the same effect [38]. In general terms, these immune responses generated after vaccination or experimental infection are punctual non-specific responses or transient (although they may last several months), and considerable variations of the cellular responses are detected amongst animals. However, a recent report detected no significant association among cattle testing positive for paratuberculosis and positive caudal fold test and the IFN-γ assay [8]. Some strategies have been applied to discriminate between a herd infected with *M. bovis* or *M. a. paratuberculosis*: (1) the use of specific mixtures (johnin, avian PPD) or specific antigens (ESAT-6, CFP-10), or (2) selection of criteria for the interpretation of the results (B/A ≥ 2). It is felt that more research should be performed to elucidate the role of co-infecting agents on the diagnosis of bovine tuberculosis [37]. To our knowledge, however, there are no reports on the use of these tests and interpretation of the results in cattle herds co-infected with both *M. bovis* and *M. a. paratuberculosis*. This situation is expected to occur occasionally in Northern and Central European countries where only small outbreaks of tuberculosis infection have occurred in recent years (as cited in [9, 26, 38]), but could be found more frequent in other countries such as in the United Kingdom, the Republic of Ireland, the

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[1] Council Directive 64/432/EEC, 1964, [on line] (2005) http://www.eu.int/scadplus/leg/en/lvb/l12004.htm#AMENDINGACT [consulted 30 January 2006].

[2] Aranaz A., del Río R., Ruiz J., et al., Effect of the paratuberculosis vaccine on the IFN-γ test for tuberculosis in goats, Abstracts, 3rd Int. Conference on *Mycobacterium bovis*, Cambridge, UK, 2000, p. 11.

Mediterranean basin countries, and some states of the USA (as cited in [8]).

This report describes a field trial conducted in a cattle herd with dual infection (bovine tuberculosis and paratuberculosis) with the objective of eradicating *M. bovis* infection. A comprehensive approach was performed using a multi-test strategy to identify *M. bovis* infected cattle. We have found that the presence of dual mycobacterial infection confound the diagnostic tests and may result in a slowing down of the progress of the eradication programmes. Furthermore, *M. bovis* isolates from domestic and wild animals from the property were characterised to determine a likely epidemiological link.

2. MATERIALS AND METHODS

2.1. Animals and design of the study

2.1.1. Herd

This study was performed in a herd of bullfighting breed cattle of outstanding genetic value (this specific stock has been inbred since the 16th century). These cattle are difficult to handle because of their vigour and temper and are bred with an extensive management system and occasional feed supplements. It is raised in a property situated in Sierra of Alcaraz (central-east of Spain). The herd had suffered from the infection of bovine tuberculosis for at least 20 years before the start of this study, with 2.7 to 4.8% of reactors (fluctuating percentages) to the IDTB test performed under official eradication campaigns. Some animals presented diarrhoea, cachexia and death (clinical symptoms compatible with paratuberculosis). The property is also exploited for game of several wildlife species.

2.1.2. Design of the study

Before the beginning of the trial, a meeting was held involving the owners of the property, the farm manager, the veterinar-

ian, the representatives of the Bullfighting Farmers Association, representatives of the regional government and the Spanish Ministry of Agriculture, Fisheries and Food, and our group to discuss the design and planning of the study. Periodic meetings after every trial allowed the evaluation of the progress.

This study includes data from all animals (females and breeding bulls) over 12 months of age that were tested in parallel by the IDTB test, the IFN-γ test and serology (ELISA test) for paratuberculosis. Since the performance of the tests was being compared on the same animals, they were tested simultaneously to avoid the interference of one test with the other [30, 41]. These tests were repeated 8 times over a 3.5-year period, avoiding the summer and the last pregnancy seasons. In total, an average of 301 (251–361) animals was tested in every trial. All animals reacting to the IDTB test, and a group of animals with positive results to the IFN-γ test and positive serology of paratuberculosis were slaughtered and true infection status was confirmed by bacteriological culture.

The rest of the cattle were allocated to two herds depending on their results: herd A, with animals negative to all tests, and their offspring; and herd B, composed of animals with at least a positive result to the IFN-γ assay or to serology of paratuberculosis (but not slaughtered), and their offspring. Herds A and B were kept in different areas of the property, with independent pastures and water resources, with no contact.

2.2. Diagnostic tests

2.2.1. IDTB test

The cervical comparative IDTB test was carried out according to the RD 2611/1996 (direct transposition of Council Directive 64/432/EEC) by the Official Veterinary Services using the official bovine and avian PPDs (CZ Veterinaria, Porriño, Spain). The animals were simultaneously inoculated 0.1 mL (0.1 mg, 2500 CTU) bovine PPD on

the left side of the neck, and 0.1 mL (2500 IU) avian PPD on the right side. The increase of skin-fold thickness was remeasured 72 h later. A positive bovine reaction, which was more than 4 mm greater than the avian reaction, or that produced clinical signs (diffuse or extensive oedema, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region or of the lymph nodes), was interpreted as a positive result to bovine PPD.

2.2.2. IFN-γ test

Blood samples were collected in tubes with lithium heparin at the time of the IDTB testing and shipped to the laboratory at room temperature. Stimulation of whole blood was performed within 8 h of collection, and 2 or 3 days were required to test the whole herd. The IFN-γ test was carried out as described [18, 29]. In the first trial, a fourth well with 2 µg/mL of poke-weed mitogen (Sigma Aldrich Gmbh, Steinheim, Germany) was included as the positive control.

Detection of IFN-γ in supernatant was performed in duplicate (in two different plates) with the ELISA kit Bovigam^{TM} (CSL Ltd, Parkville, Australia) according to the manufacturer's instructions. An animal was considered positive for bovine tuberculosis when the mean OD of the sample stimulated with bovine PPD minus the mean OD of nil antigen was greater that 0.050 [44, 45]. Other criteria previously used by other authors have been applied only to allow the comparison of the results obtained in this field trial with other scientific publications.

2.2.3. Serology of paratuberculosis

All animals were assayed in duplicate (in two different plates) using the absorbed ELISA kit Parachek^{TM} (CSL Ltd, Parkville, Australia) according to the manufacturer's instructions. An animal was considered positive when its mean OD was greater than the plate negative control value plus 0.100.

2.3. Bacteriology and molecular characterisation

2.3.1. Selection of animals and tissue sample collection

Reactors to the IDTB test and a group of other selected animals were slaughtered in abattoirs. Animals were recommended for culling if they gave positive reactions to the IFN-γ assay after whole blood stimulation with bovine PPD and/or strong positive results (OD > 0.300) in the serology for paratuberculosis. The final decision to remove animals from the herd and to segregate the animals with doubtful results was made by veterinarians in agreement with the owners taking into consideration the results of the tests and the history in previous tests (if available). Samples from a number of cattle that had not been tested were also cultured: heifers (younger than 12 months) ($n = 41$), and bullfighting bulls, approximately 4 years old ($n = 12$), that had been killed at bullfighting arenas in the years 2001 and 2003.

Samples were taken at the post mortem inspection by members of our group with the kind help of the abattoir staff. They consisted of retropharyngeal, mediastinal and bronchial lymph nodes, and lungs for detection of tuberculosis, and the ileocecal valve and mesenteric lymph node for detection of paratuberculosis (although only the ileocecal valve was collected at the first trial). All samples were stored at –20 °C until analysis.

2.3.2. Bacteriology (M. bovis)

Bacteriological culture was used as the gold standard for the determination of the true status of the animals. An animal was considered infected with bovine tuberculosis only when *M. bovis* was isolated by bacteriological culture from its tissue samples.

Tissue samples selected for bacteriological analysis were the tuberculosis compatible lesions and the adjacent area, or a pool

of the collected tissues if no macroscopic lesion was present. Smears were stained with phenolated auramine [33] and observed by fluorescence microscopy. Bacteriological culture and identification of isolates was performed as previously described [4, 42].

2.3.3. Molecular characterisation of M. bovis isolates

Thirty-six *M. bovis* isolates were characterised by spoligotyping and MIRU-VNTR: the 23 *M. bovis* isolates from the animals slaughtered in the field trial, the three isolates from bulls, and ten isolates available from wild animals.

The spacer oligonucleotide typing (spoligotyping) method [15] was performed with heat-treated cell suspensions. The biotin-labelled amplified product was hybridised onto a membrane (Isogen Bioscience BV, Maarssen, The Netherlands) and detected with the streptavidin-peroxidase conjugate (Roche Diagnostics GmbH, Penzberg, Germany) and the ECL system (Amersham Biosciences UK Ltd., Buckinghamshire, UK). A part of these results have been published previously [1].

The *M. bovis* strains were also analysed by mycobacterial interspersed repetitive units (MIRU) – variable-number tandem repeats (VNTR) aimed at loci ETR-A [10] and loci 4 [36].

2.3.4. Bacteriology (M. a. paratuberculosis)

Smears of the ileocaecal valve and mesenteric lymph nodes were stained by Ziehl-Neelsen for a presumptive detection of *M. a. paratuberculosis.* Tissue samples were decontaminated following a recommended protocol [14], cultured and the isolates were identified as *M. a. paratuberculosis* by mycobactin-dependency, colony morphology and IS*900*-PCR as described [6].

2.3.5. Wildlife sampling

Samples from wild species were also studied, including wild red deer (*Cervus elaphus*) ($n = 2$), wild boar (*Sus scrofa*) ($n = 21$), and hare (*Lepus europaeus*) ($n = 10$), all collected during the hunting season 2001–2002. Samples were cultured for *M. bovis* and *M. a. paratuberculosis* and characterised as described. The decision to separate the cattle herd from wildlife by fencing was adopted at the first meeting.

3. RESULTS

3.1. Mycobacterial isolation

After the trials, all reactors to the IDTB test and a selection of animals were slaughtered in an abattoir. In total, 143 animals were necropsied. The results from culture of 131 animals are available (Tab. I), since we could not get samples from seven animals and the culture media was contaminated in five other animals. *M. bovis* was isolated from respiratory lymph nodes and/or lungs from 23 cattle included in the field trial, 19 of the 23 showed lesions compatible with tuberculous infection.

Regarding the group of animals that were not tested, all heifers were negative, and samples from three of the 12 bullfighting bulls yielded *M. bovis*.

Paratuberculosis infection in the herd was confirmed by culture. Very slow-growing *M. a. paratuberculosis* isolates were obtained from 20 cattle. As an approach to the detection of this agent we considered an animal as likely infected with *M. a. paratuberculosis* also when acid-fast bacilli were detected in the intestinal tissue samples after direct Ziehl-Neelsen staining.

3.2. Detection of M. bovis infected cattle by the diagnosis techniques

The comparative IDTB test detected 15 (65.2%) and the IFN-γ test (bovine PPD ≥ nil

Table I. Classification of the slaughtered cattle according to their response to the diagnostic tests and bacteriological culture for *M. bovis*.

No. of animals of each status			No.	*M. bovis* culture[a]	
				Positive	Negative
44 IDTB[b] +	IFN-γ B[c]	Parachek +	1	0	1
	IFN-γ B	Parachek –	22	8	14
	IFN-γ *avian*[B][d]	Parachek +	1	1	0
	IFN-γ *avian*[B]	Parachek –	–	–	–
	IFN-γ -	Parachek +	3	1	2
	IFN-γ -	Parachek –	17	5	12
Total				15	29
87 IDTB –	IFN-γ B	Parachek +	5	2	3
	IFN-γ B	Parachek –	14	4	10
	IFN-γ *avian*[B]	Parachek +	34	1	33
	IFN-γ *avian*[B]	Parachek –	3	0	3
	IFN-γ -	Parachek +	28	1	27
	IFN-γ -	Parachek –	3	0	3
Total				8	79

[a] Results of culture are available for 131 cattle, culture not available for the other 12 animals.
[b] Comparative IDTB test performed in the neck, interpretation according to the RD 2611/1996 (direct transposition of EU directive 64/432) by the Official Veterinary Services.
[c] Bovine PPD ≥ nil + 0.050, and bovine PPD ≥ avian PPD.
[d] *Avian*[B], bovine PPD ≥ nil + 0.050, and also avian PPD ≥ nil + 0.100 and avian PPD ≥ bovine PPD.

Table II. Apparent sensitivity and false positive reactors (%) of the IDTB test and the IFN-γ assay (performed as described in the text) depending on the criteria used for the interpretation of the results.

Criteria	Sensitivity[a]	False positive reactors[b]
Comparative IDTB test	65.2	26.8
IFN-γ assay		
Bovine PPD ≥ nil + 0.050	69.6	59.3
Bovine PPD ≥ nil + 0.050, bovine PPD ≥ avian PPD	60.9	25.9
Bovine PPD ≥ nil + 0.100	43.5	31.5
Bovine PPD ≥ nil + 0.100, bovine PPD ≥ avian PPD	39.1	8.3
Bovine PPD / nil ≥ 1.5	60.9	54.6
Bovine PPD / nil ≥ 2	34.8	33.3

[a] Sensitivity determined on the 23 *M. bovis*-culture positive animals.
[b] % of false positive reactors determined on the 108 culture-negative animals (true negatives + false positives).

+ 0.050) detected 16 (69.6%) of the *M. bovis* culture-positive cattle. As expected, the number of *M. bovis* culture-positive animals detected by the IFN-γ assay decreased when less strict criteria of interpretation of the test were applied (Tab. II). The IDTB test detected infected animals during the first three trials (12 during the first trial, one

A.

B.
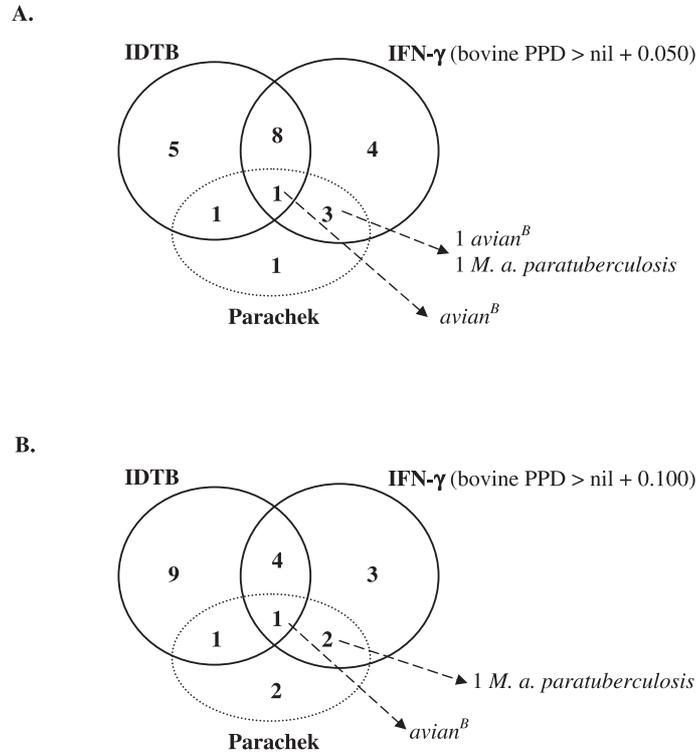
Figure 1. The results of the diagnostic tests of the 23 *M. bovis* infected cattle. The animals with unexpected results are marked. Panel A, results to the IFN-γ test were interpreted using the strict criterion (bovine PPD ≥ nil + 0.050). There was a limited overlapping of animals positive to both methods (9 out of 22, 40.9%). Two of the 16 cows that were positive to the IFN-γ assay had higher values to avian PPD than to bovine PPD (designed as *avian*[B]), both were positive to the ELISA for paratuberculosis, but both showed generalised tuberculosis lesions (one of them was classified as total condemnation at the abattoir). An animal that was slaughtered only because of its result to the serology of paratuberculosis (it was a weak avian reactor to the comparative IDTB test and negative to the IFN-γ test) was also infected with *M. bovis.* Panel B, results to the IFN-γ test interpreted according to bovine PPD ≥ nil + 0.100. Note that this panel contained only 22 cattle because one *M. bovis* infected animal went undetected. There is a reduced overlapping of animals positive to both methods (5 out of 20, 25%). The *avian*[B] reactors are also indicated. In this case, two infected animals were detected after slaughtering because of its result to the serology of paratuberculosis (not detected by IFN-γ test or the IDTB test).

during the second trial, and two during the third trial), but failed to detect infected cattle after that period. The IFN-γ test detected positive animals during the length of the study (nine, one and three in the first three trials respectively, and also one in the fourth, and two in the sixth trial). There was a very limited overlapping of the results obtained with both techniques (Fig. 1, panels A and B).

3.3. False positive reactors

Seventy-eight cattle that had been diagnosed as infected by the IDTB and/or the IFN-γ tests (Fig. 2, panel A) were negative by culture, their samples from lung and associated lymph nodes were negative by direct auramine staining, and 76 of them were no-visible lesions reactors. If a less

A.

B.
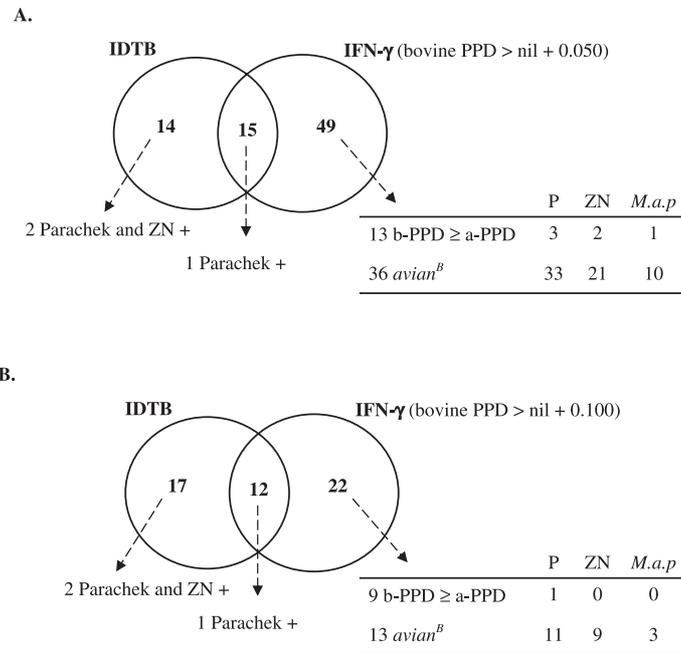
Figure 2. The results of the diagnostic tests for bovine tuberculosis of the false positive reactors (cattle positive to IDTB and/or IFN-γ test, but *M. bovis* culture negative). The results relevant to paratuberculosis infection (Parachek (P), Ziehl-Neelsen staining of digestive tissue samples (ZN) and *M. a. paratuberculosis* isolation (*M.a.p*)) are indicated. Panel A, 78 cattle are considered false positive reactors when results to the IFN-γ test were interpreted using strict criteria (bovine PPD ≥ nil + 0.050). Note the results related with the paratuberculosis infection in the group of cattle that responded only to the IFN-γ assay. Panel B, the number of false reactors decreased to 51 when the results to the IFN-γ test were interpreted using bovine PPD ≥ nil + 0.100. In this case there were still nine animals that gave a higher value to bovine PPD than to avian PPD.

strict criterion is applied (cut-off ≥ PBS + 0.100) the number of false reactors decreases to 51 (Fig. 2, panel B).

3.4. The results of the IFN-γ test obtained with mitogen stimulation

The viability of blood samples was evaluated in the first trial by non-specific stimulation of the 368 samples with poke-weed mitogen. The result of this stimulation was highly variable, ranging from no response to over the ELISA range, and the mean OD was 1.15. Only 10 animals (2.72%) showed no response against the mitogen (mitogen – PBS ≤ 0.100), and 18 (4.84%) showed a

low response (mitogen – PBS ≤ 0.200). Five of these animals were positive in the Parachek ELISA and died in the field that year. Regarding the cattle that did respond to mitogen ($n = 340$), there was a slight difference amongst the cattle positive or negative to paratuberculosis ELISA (Tab. III).

3.5. Molecular characterisation of *M. bovis* isolates from livestock and wildlife

The results of the molecular characterisation of the *M. bovis* isolates are shown in Table IV. Four spoligotypes were found, and three of them, numbered spb-7, spb-9

Table III. Non-specific stimulation of the 340 blood samples that did respond to the poke-weed mitogen.

Parachek	Response to the poke-weed mitogen[a]
Negative	1.306[b]
Positive	0.956
0.100–0.250	0.964
0.251–0.500	0.990
0.501–1.000	0.986
> 1.000	0.871

[a] Mean of the OD of the mitogen well minus the OD of the PBS well for each animal.
[b] The ELISA results *over* found only in this group were replaced with the highest value in the group to help in the calculations.

and spb-98 in our database, were isolated from animals sampled from 2001 to 2004. The isolates were divided into four types using the ETR-A typing and 2 types using MIRU-4 typing. The combination of the results of both techniques further subdivided the *M. bovis* isolates into 5 types. *M. bovis* from cattle and wild animals from the property shared the profiles of spoligotyping and VNTR.

4. DISCUSSION

In spite of the economic and logistic resources that have been devoted to the eradication of bovine tuberculosis, the prevalence of *Mycobacterium bovis* infection has not being substantially reduced in certain areas of Spain and other countries such as the United Kingdom and the Republic of Ireland. Some factors have been associated with the failure of these eradication campaigns. Firstly, the performance of the tuberculin tests could be affected by factors inherent to the method [19], or factors that have been described to impair the immune response, such as treatment with corticoids and the effect of stress [7, 12]. Secondly, some wildlife species have been blamed for acting as a reservoir. This study was undertaken to ascertain the effect of a third possibility, that it is the presence of other infections (specifically paratuberculosis) in the performance of the diagnostic tests for bovine tuberculosis. Furthermore, these situations do not rule out the existence of the others.

The results obtained in this study show that the IDTB test alone, without further analysis of results and follow-up at the abattoir of tuberculosis reactor cattle, is not the

Table IV. Results of the molecular characterisation of the 36 *M. bovis* isolates from cattle ($n = 26$) and wildlife animals ($n = 10$) obtained in the study.

Spoligotyping	ETR-A[a]	MIRU-4[a]	Animal species	Years of sampling[b]
Spb-7 (SB0121)[c]	5	4	7 cattle	2001–2003
			4 wild boar	2002
Spb-7 (SB0121)	7	3	5 cattle	2001
			1 wild boar	2002
Spb-9 (SB0295)	7	3	1 deer	2001
			2 wild boar	2001
			2 wild boar	2002
			3 cattle, 1 bull	2001–2004
Spb-34 (SB0152)	8	3	1 cattle	2001
Spb-98 (SB0933)	4	3	7 cattle, 2 bulls	2001–2004

[a] Number of copies.
[b] Wildlife animals were sampled only in 2001 and 2002.
[c] SB0121 (HEX code 6F-5F-5E-7F-FF-60), SB0295 (6F-5F-5E-7F-FF-20), SB0152 (40-00-00-7F-FF-60), and SB0933 (60-5F-5F-7F-FF-40) are the number of the patterns as found in the *M. bovis* spoligotype database (www.Mbovis.org).

optimum strategy for the eradication campaign if both mycobacterial infections are present. It presents two main drawbacks in the eradication campaign; first, the unsatisfactory level of sensitivity to clear the infection, and secondly, the finding of false tuberculosis reactors, which is detrimental to farmers, results in serious financial loss and may lead to non-compliance with eradication schemes.

The development of the IFN-γ test has been a major advancement in the diagnosis of bovine tuberculosis and it has been officially approved in many countries. However, there is little information about the performance of this test and the criteria that should be used in the different epidemiological contexts. Since our main target was the eradication of tuberculosis in the herd, the cut-off value of the ELISA for IFN-γ was adjusted to obtain a maximum sensitivity (bovine PPD ≥ nil + 0.050).

Although it is difficult to compare the results obtained in the published field trials because of the use of different methodology (sampling time, different PPD and ELISA kit), source of cattle, and criteria of interpretation, the sensitivity values obtained in this study for both the IDTB and the IFN-γ tests have been only moderate, and in general terms, they were lower than those found in other reports [13, 18, 28, 31, 32, 45]. This low performance is more obvious regarding the IFN-γ assay since it is generally accepted to have a higher sensitivity. In this study, the apparent sensitivity of both IDTB and IFN-γ tests was low and similar, 65.2% and 69.6% respectively. The sensitivity of the tests may be overestimated because only a moderate number of non-reacting cattle were necropsied. As reported in other studies, there was an overlapping population of infected animals that was detected by both the IDTB test and the IFN-γ assay, and a population positive on either the skin test or the IFN-γ assay. In this case, this common population was also small (40.9%). Therefore, and in agreement with other authors [13, 28, 32, 39, 45], the

detection of the maximum number of infected animals is achieved by the combination of both tests. Both tests were complementary also in the way that the IDTB test performed better during the first part of the trial, while the IFN-γ test was the only method that detected the infected animals at the second part of the study (it detected the *M. bovis* culture positive cattle that failed to react to the IDTB test). The practical effect in the eradication of this infection is that by using the comparative IDTB test alone, the herd would have recovered the officially tuberculosis-free herd status that enable for movement and trade of animals although some residual *M. bovis* infection remained at that moment. Cattle movement and cattle purchase is a potential for the transmission of the disease [27]. In fact, a recent paper highlights that the movement of cattle from areas where bovine tuberculosis is reported outperforms other variables as the predictor of disease occurrence in Great Britain [11].

Two of the *M. bovis* culture positive animals were *avian*[B] reactors. The identification of avian reactors further complicates the interpretation of the results. These animals are usually considered negative to tuberculous infection, and it has been reported that cows with a positive IFN-γ response to avian PPD generally also had positive lower responses to bovine PPD [35]. However, the isolation of *M. bovis* from *avian*[B] reactors was described in a goat flock [18]. We have found that it can also occur in cattle.

Serial testing combining two techniques (IDTB and IFN-γ assay) has been previously used for the eradication of tuberculosis in goats[3] [18] and cattle [13] resulting in

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[3] Vidal D., Domingo M., Aranaz A., et al., Eradication of tuberculosis from goat herds by means of the interferon-γ assay and the single intradermal comparative skin test, in: Griffin F., De Lisle G. (Eds.), Tuberculosis in wildlife and domestic animals, Proc. 2nd Int. Conference on *Mycobacterium bovis*, Otago Conference Series, University of Otago Press, Dunedin, New Zealand, 1995, 328–330.

gradual reduction in the prevalence of the infection and elimination after two to four cycles. The fact that more cycles were needed in this herd could be due to the type of cattle, the presence of paratuberculosis in the same herd, and a possibility of infection from wildlife species.

Although the use of a mitogen as the positive control has not been considered absolutely necessary [37], we included stimulation with poke-weed mitogen as a positive control, and the general immune response obtained was satisfactory. The results indicate that the response of cattle negative to the Parachek test is slightly higher than those positive to the Parachek test. Secretion of greater quantities of IFN-γ in response to mitogens in cows with subclinical paratuberculosis than in cows with clinical paratuberculosis has been reported [34]. The finding that five of the cattle which did not respond to mitogen were at the last stage of paratuberculosis infection may suggest that clinically advanced paratuberculosis hampers the development of cell responses, resulting in false negative reactions.

Although our data should be interpreted as approximate because the slaughtered animals were not randomly selected, the number of false reactors (IDTB test positive and/or IFN-γ test positive, but *M. bovis* culture negative) found in this study was remarkably high compared to other reports, with the exception of another Spanish study [13]. Our results were obtained on a single farm, but we think that they could be extrapolated to many other farms in the same singular epidemiological situation.

In our experience, the number of false positive reactors is too high to be explained only as a failure to detect *M. bovis* because (1) lesions were in unusual location and therefore not sampled at the abattoir because we examined the tissues with a higher probability to contain bacilli (mediastinal, retropharyngeal, bronchial and mesenteric lymph nodes, and lung) [5, 40], or (2) as failure of the bacteriological cul-

ture to detect low-viability bacteria that could be damaged after freezing or decontamination procedures [5]. In total, 39 of the 78 (50%) false reactors were positive in the serology of paratuberculosis using the Parachek kit, 24 were positive in the Ziehn-Neelsen staining, and *M. a. paratuberculosis* was isolated from 11 cattle; most of them were from the group detected by the IFN-γ assay. Therefore, we think that this can be explained by the cross-reactivity due to bacteria other than *M. bovis*, likely cross-reactivity due to *M. a. paratuberculosis*.

The animals were separated into two herds according to their results to the tests. The advantages of this systems of segregation (apart from the study of the evolution of the results) are that it avoids unnecessary culling and helps to maintain a number of cattle in the property to keep its economic viability, while limiting the spread of the infections. Only the offspring of herd A were used for replacement, and as the control progressed, the trend was the elimination of herd B. The obvious drawback is that it can be carried out only when no limitation of land and other resources is present.

Another aspect of the epidemiology of the *M. bovis* infection that was taken into account in this study was the potential role of wildlife as reservoirs of the infection. *M. bovis* can also infect a wide range of domestic and wild animals [21, 25] and some of them are considered as reservoirs of infection for livestock. The risk these reservoirs constitute for domestic animals depends on the specific epidemiological situation of the species and the environment [21] and has been demonstrated under a similar situation in Spain [1].

Sustainable control policies can only be achieved through a better understanding of the epidemiology of tuberculosis in both cattle and wildlife reservoirs [2]. The *M. bovis* isolates obtained in this study were characterised by spoligotyping and VNTR typing, and the results indicate that three of the five patterns are shared by domestic and wildlife species in the property. The awareness of

this potential risk resulted in a more precise targeting of control measures; the owners were advised to take measures to limit contact between cattle and wild species, and wildlife was fenced in a separate area.

For the time being, the strategy described in this report has been applied for 3.5 years in the herd, and we have not found any *M. bovis* culture-positive cattle since the sixth trial. All animals in herd A were negative to the IFN-γ assay in the last trial to date. The herd has achieved the officially tuberculosis-free status, but continuous surveillance will be maintained.

In summary, we found that the performance of the IDTB and the IFN-γ tests is impaired in cattle with dual mycobacterial infections; and this drop is more evident in the latter. In this context, it is not possible to accurately forecast the infection status of an animal with a single test. These results indicate that a different mentality is required in approaching the eradication campaign in herds where both diseases are present. The combined use of diagnostic techniques allied to improved farm management practices have been useful for the objective of this field trial. The strategy implemented in the property (serial combined use of diagnosis techniques and segregation of animals depending on the results, together with separation of domestic and wild animals) has resulted in the elimination of *M. bovis* infection from the herd and a significant control of the paratuberculosis infection. However, in especial when a herd is dually infected, these procedures require a substantial technical and economic effort both in farm management and in the laboratory, because a detailed follow-up of the animals is needed. These strategies should be understood as a medium-long term task, and the trusted collaboration of all implied parties is essential.

ACKNOWLEDGEMENTS

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