Validity of intradermal tuberculin testing for the screening of bovine tuberculosis in Madagascar

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ABSTRACT

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A sample survey with the objective of determining the prevalence of bovine tuberculosis by means of an intradermal tuberculin test was conducted in Madagascar and it was found that the prevalence rate varied from 0–30 % by veterinary district. In order to estimate the true prevalence, the validity of the test was investigated by assessing its sensitivity and specificity in two groups of animals from two different regions, which were destined for slaughter. In the first group where the probability of non-infected animals should have been the highest, sensitivity was estimated at 0.52 (n = 21) and specificity at 0.99 (n = 79). In the second group selected on the basis of apparent ill health of the animals in a high-prevalence bovine tuberculosis area, sensitivity was estimated at 0.8 (n = 10) and specificity at 1 (n = 12). The results obtained from both groups of cattle were not combined for statistical purposes because the sensitivity of the skin test seemed to fluctuate in relation to the chronicity of the disease. These fluctuations are discussed. However, since the first group of zebu cattle was more representative of the cattle population across the country as a whole, its results were retained as operational parameters for further screening.

Keywords: Bovine tuberculosis, cattle, chronicity, testing, tuberculin, Madagascar

INTRODUCTION

Bovine tuberculosis (BTB) is the main cattle disease in Madagascar and its prevalence evaluated in a sample survey throughout the country, varied from 0–30 % by veterinary districts.

The economic importance of BTB in Madagascar was described by Blancou & Cheneau in 1974 but it also constitutes a public health problem, less well documented than elsewhere (Blancou, Rakotoniaina & Cheneau 1974; Rasolofo-Razanamparany, Menard, Rasolonavalona, Ramarokoto, Rakotomanana, Auregan, Vincent & Chanteau 1999).

Control measures of BTB used to be applied in the 1960s and up to the beginning of the 1970s (Blancou, Rorhbach, Perdrix, Choquel & Rosner 1971) but the programme thereafter ceased and no active steps to control it were performed until 1996 when a national programme was set up with the objectives of determining its distribution and implementing control measures. The programme encompasses a cross sectional prevalence survey and an economic study which may provide data to fit control strategies according to socio-economical parameters. However, the evaluation of the true BTB prevalence (Pr) de-

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pends on the validity of the intradermal tuberculin test that is used and on the observed prevalence (Rogan & Gladen 1978) estimated in the sample survey.

According to the method used, the potency of the tuberculin and the different criteria for the determination of true negatives and true positives, the sensitivity (Se) and specificity (Sp) of the caudal fold, single intradermal cervical and comparative cervical tests, vary from 0.32–0.96 and 0.74–0.999, respectively (Francis, Seiler, Wilkie, O'Boyle, Lumsden & Frost 1978; Benet 1990; Monaghan, Doherty, Collins, Kazda & Quinn 1994; O'Reilly 1997). Consequently, mean values for Se and Sp cannot be used in an approximation to correct the observed prevalence rates and it became necessary to evaluate Se and Sp under local conditions (O'Reilly & Mac Clancy 1978). These two characteristics are constant across different populations with different prevalences: internal validity of the test (Rogan & Gladen 1978; O'Reilly 1995).

The immune response in bovine tuberculosis occurs within 3 to 6 weeks after infection (Francis 1958). However, the immune response in some categories of animals can become undetectable using tuberculin (Kleeberg 1960; Lepper, Pearson & Corner 1977). Studies in developing countries are scarce although it is known that differences in the epidemiology of the BTB in tropical conditions have an effect on Se and Sp (Kleeberg 1960). The presence of atypical mycobacteria seems to be high in tropical areas (Worthington 1967), and these organisms may contribute in reducing the specificity of the tuberculin test in cattle. On the other hand, cattle with old inactive lesions, or the advanced stage of the disease in an organ (e.g. advanced pulmonary or mammary gland tuberculosis) may result in low test sensitivity and, occasionally, the absence of a significant intradermal response (anergy). Low skin sensitivity or anergy to bovine tuberculin may be frequent in Madagascar where no preventive measures have been practised for many years.

The objective of this study was to determine the validity of the intradermal tuberculin test for cattle in Madagascar by comparing field results with laboratory results which are registered as the "gold standard". An infection by *Mycobacterium bovis* (*M. bovis*) leads to the development of a delayed-type hypersensitivity reaction which in animals injected intradermally with tuberculin, results in a response which is at its maximum 72 hours after injection. The true status of infection is evaluated on tissue samples collected *post mortem* and examined for the presence of *M. bovis* using appropriate cultural and identification techniques. A presumptive diagnosis of bovine tuberculosis in culture-negative animals with gross TB-like lesions can be made by histopathological examination of haematoxylin and eosin stained sections of such lesions.

The results obtained are analyzed and possible strategies of testing cattle for BTB in countries without active control are discussed.

MATERIAL AND METHODS

Experimental animals

The intradermal tuberculin tests were carried out on zebu cattle reaching the abattoir. It was therefore possible to collect tissue samples on carcasses after slaughtering of the animals.

A group comprising 100 animals (group 1) was selected randomly from a licensed export abattoir for the European Union (EU). Cattle for export are purchased by abattoir operators from livestock markets and are selected according to their external healthy appearance. According to EU regulation, no animal reacting to the intradermal tuberculin test may enter an export abattoir and tuberculin testing is not practised in Madagascar, except for export market. For economic reasons, animals are therefore selected by abattoir operators in low prevalence BTB areas. Consequently, animals in group 1 represent a group which should have the lowest *M. bovis* infection that it is possible to find in Madagascar. Such a group was necessary to obtain the best assessment of the intradermal tuberculin test specificity.

Animals which react positively to the test in the export abattoir selection process are nevertheless sold for local slaughter. Those reactors discovered in group 1 were traced and inspected after they had been slaughtered in a traditional abattoir.

Group 2 comprised 36 cattle reaching a traditional abattoir in an area in which prevalence of BTB is high. This area was selected in order to maximize the proportion of infected animals (true positives), in order to improve the estimate of test sensitivity. Animals in this area were selected for tuberculin testing on the basis of clinical examination suggesting possible external signs of tuberculosis such as leanness, weakness, enlarged superficial lymph nodes and coughing. The relative low number of animals in group 2 as compared to group 1 was due to cattle owners fear to collaborate with us because of the risk of condemnations of complete or part of the carcasses as a result of thorough post mortem inspections.

Although it was desirable that the cattle tested would be as representative of the cattle population as possible with regards to sex, breed and age group, this objective could not be achieved. Government regulations only allow males to be slaughtered.

Tuberculin tests

Intradermal cervical tuberculin tests were performed on all the animals in both groups according to stand-

ard procedures and were read after 72 hours both subjectively by palpation of the injection site and observation of clinical signs, and objectively by measuring with a calliper rule any increase in skin-fold thickness. European Union norms were used as reference (Directive 80/219/EEC).

In order to select optimal interpretation criteria, a plot was made of the true positive rate (sensitivity) against the false positive rate for the different possible cut-off points for the objective test. Receiver Operating Characteristic (ROC) curves were built in order to determine the best level of skin-fold thickness increase to be used for a screening test.

In group 1, the intradermal tuberculin test was performed simultaneously using two bovine tuberculins (Tuberculine, Imvavet and Tuberculine Bovine PPD, Fort Dodge Santé Animale), the first being a local product and the second being imported. The objective was to assess the validity of the test with both tuberculins and to determine the efficacy of the local product for possible future use. One of the products was injected into one side of the neck and the other on the opposite side. An avian PPD tuberculin (Avituber, Merial) was also injected in the cervical region. Doses of 0.1 mℓ were used for both the Tuberculine Bovine PPD containing 20 000 Community Tuberculin Units/mℓ (CTU/mℓ)) and Avituber containing 25 000 IU/mℓ. A dose of 0.2 mℓ containing a total 5 000 Community Tuberculin Units was used for the local bovine PPD tuberculin according to the manufacturer's instructions. Community Tuberculin Units and International Units of bovine PPD tuberculin are biologically equivalent (Haagsma 1997).

In group 2, the animals were tested with Tuberculine Bovine PPD and Avituber using the same procedures and dosage rates as used in the first group.

Necropsy and tissue sampling

In both groups, the animals were identified with an individual paint mark on the skin, and the injection sites were also identified by means of circles of paint around each site.

After the animals were slaughtered, the main organs, viscera and most of the lymph nodes of each carcass were inspected. Enlarged lymph nodes with suspected BTB lesions were collected and taken to the laboratory without opening them to avoid external contamination. In the absence of large gross lesions, lymph nodes were sliced to look for small lesions (primary complex). In animals in which lymph nodes were normal in size and BTB lesions were not suspected, three pairs of those organs most frequently affected in *M. bovis*-infected animals, i.e. the right and left retro-pharyngeal, right and left tracheo-bronchial and mediastinal cranial and caudal lymph nodes, were collected for culture, without incising them in order to reduce possible contamination.

Laboratory analysis

The "gold standard" adopted in this trial was a combination of two different laboratory procedures. Material obtained from gross lymph node lesions and from the lymph nodes most frequently infected was cultured on Löwenstein Jensen (LJ) medium and LJ plus pyruvate medium according to international bacteriological guidelines (Grange & Malcolm 1994). All such tests were performed at the Institut Pasteur de Madagascar, Antananarivo.

When a culture had remained sterile for 3 months, it was declared negative. Histopathological examinations were performed only on culture-negative / lesion-positive samples to confirm or eliminate tuberculosis. Although it was not a definitive finding, histopathological diagnosis of BTB (presumptive *M. bovis* infection) was based on the findings of characteristic tubercles with or without caseation necrosis.

A test was declared positive if *M. bovis* was cultured or if characteristic tuberculous lesions were detected on histopathological examinations. The confirmation of tuberculosis on the basis of histopathological findings, did not identify the species of mycobacteria involved, however *M. bovis* is the most probable cause, not only because of its pathogenicity, but also because the predictive value for a true positive (*M. bovis* infection) histopathological finding is directly related to the prevalence of BTB.

RESULTS

Group 1

The single intradermal bovine tuberculin test performed on 100 animals was positive in 12 of them (Table 1). However, tests using both bovine tuberculins were not always positive in the same animal. Concordance observed between the two tests has been measured by the kappa coefficient: K = 0.905. Nevertheless, the tuberculin test done with Tuberculine Bovine PPD was considered as the reference because this biological reagent has been tested according to EU procedures.

Twenty-one cattle in the 100-animal sample were declared infected. True prevalence (Pr = 0.21) was higher than test prevalence (Pr o = 0.12).

Ten infected animals did not react to the tuberculin test (anergy) which represented 10 % of the initial sample and 47.6 % of all infected animals.

The mean percentage of *M. bovis*-infected animals, non reactive to the tuberculin test without macroscopical lesions and positive for *M bovis* on culture, was 4 % which represents 19 % (4/21) of infected animals. The incidence of infection (I_c) in this sample, calculated on a contact period between the ani-

TABLE 1 Tuberculin test versus reference test results in the evaluation of test validity in cattle in Madagascar (group 1)

Gold Standard: culture +/– histopathology						
Tuberculin Test	Infected			Non infected		
	Culture[a] (lesion[b])	Histopathology[c]	Total[d]	Culture[e] (lesion)	Histopathology	Total[d]
+	9 (9)	ND	9	0 (0)	ND	1
	0 (2)	2	2			
-	7 (3)	ND	7	0 (0)	ND	77
	0 (3)	3	3	1 (0)	ND	1

[a] number of *M. bovis* isolated from cultures in the samples
[b] number of lesions related to the samples
[c] number of lesions analyzed by histopathology
[d] total number of reactions according to culture and histopathological status
[e] number of atypical mycobacteria isolated

TABLE 2 Sensitivity and specificity of the tuberculin test done with subjective measure according to tuberculin and sample

Group number (Tuberculin)	Specificity			Sensitivity		
	Value	N	CI	Value	*n*	CI[a]
1 (Tuberculine)	0.99	79	0.96 1.00	0.52	21	0.30 0.74
1 (Tuberculine Bovine PPD)	0.99	79	0.96 1.00	0.52	21	0.30 0.74
2 (Tuberculine Bovine PPD)	1.00	12	0.74 1.00	0.80	10	0.44 0.98

[a] Confidence Interval calculated without approximation (binomial method)

TABLE 3 Tuberculin test versus reference test combined results in the evaluation of test validity in cattle in Madagascar (group 2)

Gold Standard: culture +/– histopathology						
Tuberculin Test	Infected			Non infected		
	Culture[a] (lesion)	Histopathology[b]	Total[c]	Culture[d] (lesion)	Histopathology	Total[c]
+	8 (8)	ND	8			0
-	1 (1)	ND	1	– (0)	ND	11
	– (1)	1	1	1 (0)	ND	1

[a] number of *M bovis* isolated from cultures
[b] number of lesions analyzed by histopathology
[c] total number of reactions according to cultures and histopathological status
[d] number of atypical mycobacteria isolated

mals estimated at 1 month before slaughtering, is: I_c = 58/100 animals-year.

Subjective evaluation of the single intradermal test on the basis of the presence or absence of BTB by clinical evaluation of test animals and palpation of the cervical sites of injection 72 h following the injection of cattle gave the same results with both bovine tuberculins (Table 2).

A ROC curve was drawn from the results read by skin-fold thickness increase by the objective technique with Tuberculine Bovine PPD (Fig. 1A).

Concordance of the results obtained with both tuberculins can be evaluated by kappa coefficient. Different skin-fold thickness levels were tested and gave the following results: $K_{L>4\,mm} = 1$, $K_{L>5\,mm} = 0.9$ and $K_{L>1.5\,mm} = 0.795$. Kappa coefficient provided best results for 4 mm level, but lack of data did not allow the testing at levels between 1.5 and 4 mm.

Reaction to avian tuberculin occurred in three animals but only in the absence of a bovine tuberculin reaction. In one such case, no lesions were found; in the two other cases, lesions were present in the lymph nodes of the carcasses, but no bacterial growth

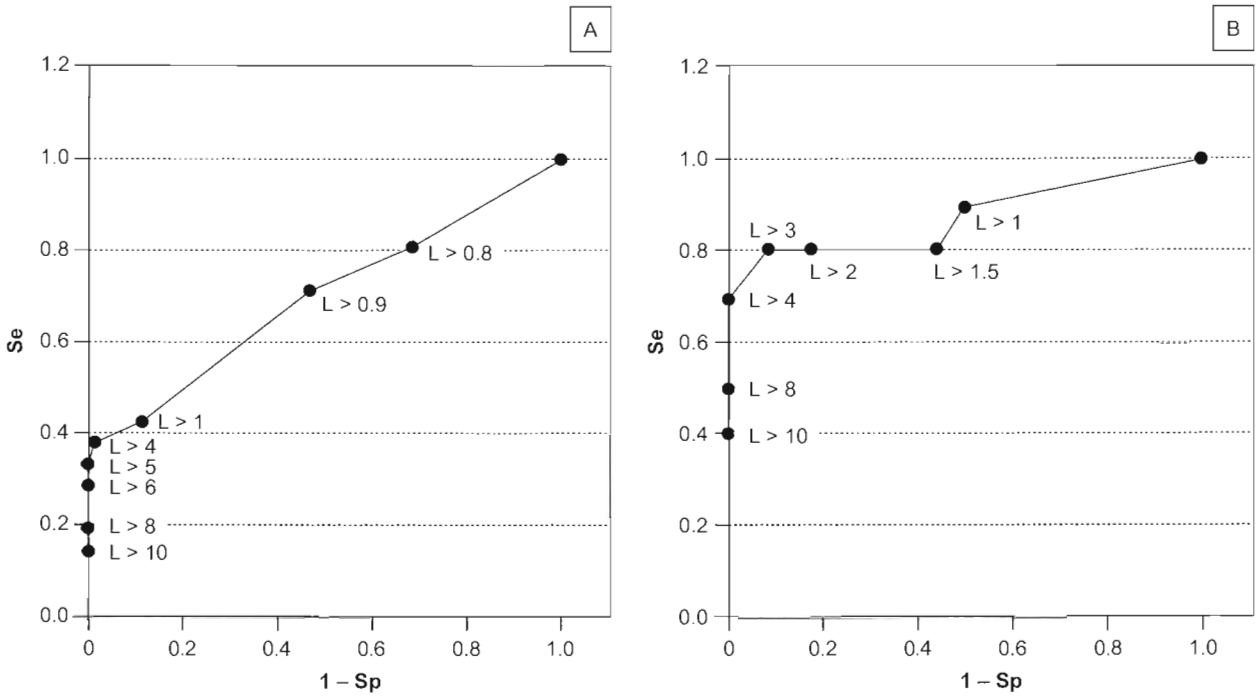

FIG. 1 ROC curve for the determination of the best skin-fold thickness increase level (L) (mm) to be used for the tuberculin test (Tuberculine Bovine PPD)

A Group 1 = representative of the general population
B Group 2 = issued from a high prevalence area

was obtained in those cultures. Histopathological examination of them revealed the presence of tuberculosis-like lesions, and therefore these animals were considered as infected. Conversely, one atypical mycobacterium was isolated in the absence of a lesion and without the animal reacting to the bovine and avian tuberculin tests.

Group 2

Only 22 cattle of the initial sample of 36 were presented for reading the skin test and subsequently slaughtered. It was impossible to gather further information concerning the other 14 which were missing and were slaughtered outside of the control of the Veterinary Services.

Eight of the 22 animals reacted to the skin test, all of which were infected (Table 3). Ten of the 22 animals were declared infected (45.5 %), nine exhibiting gross tuberculous lesions which yielded *M. bovis* on bacteriological culture. One, bacteriologically negative, was declared positive following histopathological examination.

The results of the subjective evaluation of the tuberculin tests, read 72 h after injection, are summarized in Table 2. The results of the sensitivity and specificity tests read by measuring the skin-fold thickness are presented in Fig. 1B.

In this group, two cases reacted to both the avian and the bovine tuberculin. These were considered to be positive for *M. bovis*. Another animal reacted to the avian tuberculin test but not to the bovine one, no lesions were found and the culture remained sterile. This animal was classified as non-infected. One animal developed a slight reaction to avian tuberculin, however it did not react to bovine tuberculin but a lesion was detected. Bacteriological examination of the lesion was negative, although on histopathological examination it was diagnosed as being tuberculosis-like. This animal was considered to be infected. One atypical mycobacterium was isolated from an animal in the absence of lesions; it did not react to either the bovine or avian tuberculin tests.

DISCUSSION

Effects of the animal selection process and the absence of control measures for BTB on the parameters obtained

In any trial designed to compare doses of tuberculin in cattle, there are definite economical and biological advantages to carry out each comparison on the same animal. However, experiments in Australia in BTB free beef cattle artificially sensitized with killed *M. bovis* showed that the simultaneous injection of 0.1 mℓ doses of bovine PPD tuberculin severely de-

pressed skin sensitivity as compared with control cattle injected only with unique doses (Lepper & Corner 1976). If this finding is a true reflection of the situation in which animals with naturally acquired *M. bovis* infection are injected simultaneously with more than one bovine tuberculin, then skin sensitivity will be also depressed leading to an underestimate of test Se. This possibility cannot be ignored in this trial in Madagascar.

Se and Sp were calculated for both samples. Se estimates are not very accurate because of the lack of infected animals. A non-significant difference was observed for Se for the test made on the two groups by subjective technique and the results should have been pooled in order to increase the power of the estimate. However, the non-significance of the difference observed in values of Se between the two groups (0.52 vs 0.8) seems to be related to a lack of power due to low numbers of true positives, particularly in group 2.

Immunological aspects can explain this difference. Group 1 included infected animals which remained below the tuberculin test skin reaction detection level. They presented as animals with old lesions which were less immunogenic, or as animals with old, large and extended lesions which had overwhelmed the immune reaction capacities of the animals. The same findings were observed in field trials in Australia. In a group of 31 *M. bovis* infected cattle identified as false negatives to the caudal fold tuberculin test, six had generalized lesions of tuberculosis, while the remaining 25 had lesions in only one isolated lymph node (Francis *et al.* 1978).

The incidence rate of recently infected animals in group 1 (58/100 animals-year) was exceptionally high. These animals were probably infected during the gathering and herding process on the way to the abattoir (usually about 1 month) and as they were in the pre-allergic phase of infection, had not yet developed their immune response.

These two groups of animals constituted of non-responders led to a reduction of test Se.

On the other hand, in the study area of group 2, animals are commonly sent to the slaughterhouse when suspected to be diseased in order to reduce further economic losses. Thus, old inactive lesions are likely to be less frequent in this group. Recently infected animals should be scarce because they were gathered only a few days before being slaughtered. As a consequence, the logistics of the selection of the two experimental groups of animals (groups 1 and 2) led to a bias in the calculation of the validity of the test, because the groups of animals were not at the same stage of the infection or disease. Consequently, Se should be higher than in group 1.

Furthermore, other biases due to the animal selection process are impossible to control since slaughtering females and young animals is not allowed in Madagascar. Kleeberg (1960) also described false positive reactions in oxen.

In the single intradermal tuberculin test, validity parameters should be calculated for the spectrum of immune responses related to stages of infection or disease (i.e. recent infection, and long standing active disease or old lesions) and geographically according to the fluctuation of prevalence rates across the country. The wide variations in test Se in groups of chronically infected animals and recently infected animals depend on the absence of control measures for BTB for many years. This situation is unknown in the countries where the test and slaughter policy has been applied for a long time but is a reality in practically all the African countries because very few have active control programs for BTB. Consequently, the determination of the validity parameters in each situation is impossible.

Values for local parameters of skin test

The values of the parameters retained for Madagascar in a first approximation were those from group 1 (Se = 0.52; Sp = 0.99; Tuberculine Bovine PPD), where animals were more likely to be compared with the general cattle population across the country. A similar low value for Se was described by Benet (1990). Sp value was high which is common in high prevalence areas where non-specific or cross-reactions are rare.

One can suppose that the value for Se calculated on apparently healthy animals (group 1) should be slightly lower than in the general population which contains animals like those from group 2 and where the rate of recently infected animals is lower. Correction of observed prevalence rates with low value of Se obtained in group 1 leads to a slight overestimation of true prevalence rates.

Avian tuberculin was only used to give an orientation in the case of cross infection with atypical mycobacteria, since they are held responsible for interfering with *M. bovis* in tuberculin tests (Worthington 1967).

Results read by skin-fold thickness increase on a ROC curve (Fig. 1A) do not provide good results for Se with acceptable values for Sp. When Se reaches 0.5, Sp decreases drastically and values for skin-fold thickness increase are not suitable (around 1 mm). The inclusion of subjective observation of clinical symptoms leads to a better estimation than only the measure of skin-fold thickness. In group 2, ROC curve (Fig. 1B) seems to be more suitable but it should be confirmed with more field data. Levels of skin-fold thickness increase are similar to those described in EU recommendations (between 3 and 4

mm) and provide a sensitivity close to subjective assessment and also a good specificity.

Influence of atypical mycobacteria

The influence of atypical mycobacteria is difficult to evaluate because only two samples gave positive cultures, both in the absence of lesion. Three animals presenting gross lesions on post-mortem examination reacted to the tuberculin test done with avian tuberculin in the absence of reaction to bovine tuberculin. The lesions were sterile and the animals classified as infected. In this case, infection by *M. bovis* may have been wrongly concluded. PCR techniques should help to determine the species of mycobacteria involved in these infections.

Negative cultures occurred in three tuberculin test negative/ lesion positive animals in which infection was suspected on the basis of the 'Gold Standard' histopathology. This leaves open the possibility that the lesions may not have been due to an infection with *M. bovis*, but to atypical infection. On the one hand this leads to an underestimation of True Negatives and test Sp and on the other hand an overestimation of True Positives and therefore test Se. However, atypical mycobacteria do not seem to be a very important problem in Madagascar since skin reactions to avian tuberculin does not occur frequently in doubtful cases and skin reaction to bovine tuberculin never occurred when an atypical mycobacterium was isolated. A preliminary study in the same conditions showed a high rate of atypical mycobacteria in non-reactive animals without lesions. The hypothesis of contamination at the abattoir or at the laboratory was suspected and preventative measures in sample collection implemented. This contamination seems to be common (Worthington 1967).

Strategy proposed for countries with high prevalence rates of BTB

The objective in determining the validity parameters of the intradermal tuberculin test was to evaluate the true prevalence of the disease across the country based on the observed prevalence in order to fit control strategies for the disease.

The low sensitivity obtained could be improved using classical techniques (repeated testing after eliminating diseased animals, using stronger tuberculins). The Interferon-g test could also provide a higher sensitivity but it must be first evaluated in the same conditions. Animals with long standing disease could also not react as for the intradermal test. In addition, this test is not well adapted for the screening in the field because it implies strong logistics to be able to transfer blood samples to a laboratory having the capacity to prime, incubate T cells and run an ELISA within 24 h after bleeding the animals.

The intradermal tuberculin test provides underestimated rates for prevalence. Using the lower value of sensitivity (0.52), the true prevalence would be overestimated across the country as a first approximation. Despite its limits, the intradermal tuberculin test could nevertheless be used first to detect infected herds instead of being used at the individual level.

In high prevalence rate areas, it is impossible to use the 'test and slaughter' policy for evident economical reasons in developing countries. Hence, other methods like vaccination should be evaluated to try to reduce the transmission of *M. bovis* in cattle populations and to achieve first low prevalence levels. This method implies the evaluation of possible contamination of humans in close contact with cattle, and the study of further detection of infected and/ or vaccinated animals. In any case, clinically ill animals must be eliminated.

After the expected reduction of the prevalence observed following a control campaign, the validity parameters of the intradermal tuberculin test are likely to be increased and will have to be re-evaluated for screening purposes. At this time, it will be possible to evaluate the cost-benefit of using the test and slaughter policy based on a reasonable low level of the disease across the country.

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