



EUROPEAN COMMISSION
HEALTH & CONSUMERS
DIRECTORATE-GENERAL
Directorate D – Animal Health and Welfare
Unit D1- Animal health and Standing Committees

**EUROPEAN REFERENCE LABORATORY (EU-RL)
FOR BOVINE TUBERCULOSIS
WORK-PROGRAMME 2011
PROPOSAL – Version 2**



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Preamble

The work-programme of the European Union Reference Laboratory (former Community Reference Laboratory) for bovine tuberculosis during 2011 is based on the actions planned for five years that were designed to fulfill the responsibilities for Community Reference Laboratories described in the Article 32 of Regulation (EC) No 882/2004 and the additional responsibilities and tasks laid down in Annex II to Commission Regulation (EC) No 737/2008 of 28 July 2008.

This current programme follows the one designed for 2010 in both the outline and description of tasks including new activities suggested by the Commission (ie. diagnosis of tuberculosis in camelids). Tasks described in this programme will be performed in consultation and collaboration with the Commission and Member States. A degree of flexibility should be understood to suit the needs of the Commission and the Member States arising during this period.

Introduction

Bovine tuberculosis is an infectious disease caused by microorganisms of the *Mycobacterium tuberculosis* complex. This mycobacterial infection represents a major concern worldwide because of its high economic impact due to mortalities, condemnations, decreases in productions, and its zoonotic potential.

For almost a century, eradication of bovine tuberculosis has been a major objective of farming communities and public authorities. Current EU policies on eradication are best understood after considering the progressive development of relevant Community Legislation.

Eradication programmes for bovine tuberculosis based on a test-and-slaughter policy have been implemented for many years in the European Union. This scheme proved to be successful in some countries, however, has been unable to eradicate the infection in others despite the use of vast economical and human resources. Eradication schemes have been successful in some countries (Denmark, Netherlands, Finland, Sweden, Germany and Luxembourg, Austria, some regions in Italy, France, Belgium, and Czech Republic) although the situation in the other Member States with eradication campaigns co-financed by the Commission is variable, reflecting differences in husbandry systems and environmental conditions. Currently, studies are undertaken to define the causes of this situation in these countries regarding: a) contribution of cattle-to-cattle transmission at the same area (neighbouring farms and communal pastures) or after movement of animals; (b) role played in the epidemiology by other domestic animals and wildlife; and c) the effect of sensitivity of diagnosis tests and potential interferences in the results.

At the moment there is a general concern regarding implementation of control programs or developing diagnostic tests in other animal species as for example goats, camelids or zoo animals. Therefore, there is a need to compare and develop new reagents suitable for the detection of infected animals in animal species other than cattle.

The working plan outlined in this proposal aims at the implementation of specific tasks by the European Union Reference Laboratory (EU-RL) for bovine tuberculosis devoted to: (1) maximise the interactions between Member States through active collaboration; (2) harmonise the protocols across countries of the European Union; and (3) contribute to the scientific, technical, and policy objectives proposed by the Commission.

The objective of the work-programme is to cover the five points of Annex II to Commission Regulation (EC) No 737/2008:

1. To coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing bovine tuberculosis; and
2. To facilitate the harmonization of techniques throughout the Community, in particular specifying standard test methodologies.
3. To organize workshops for the benefit of national reference laboratories as agreed in the work-programme and annual budget referred to in Articles 2 to 4 of Regulation (EC) No 156/2004, including training of experts from the Member States and, as appropriate, from third countries, in new analytical methodologies.
4. To provide technical assistance to the Commission and, upon its request, to participate in international for a relating to the diagnostic of bovine tuberculosis, concerning in particular the standarisation of analytical methods and their implementation.
5. To perform research activities and, whenever possible, co-ordinate research activities directed towards the improved control and eradication of bovine tuberculosis.

1. Main activities of the EU-RL for Bovine Tuberculosis for 2011.

1.1. Potency test of tuberculins.

A key task of the EU-RL will be the potency studies of all tuberculins (Purified Protein Derivative, PPD) and antigens submitted by National Reference Laboratories. These reagents are basic to immunology-based tests that are used for *in vivo* diagnosis. Skin tests are the main techniques used worldwide as the official diagnostic tests in the eradication programmes and large differences among potencies have been described depending on manufacturer and batches.

The potency of tuberculins will be assayed in cattle, since they are the animal species where they will be used in the eradication campaigns. In cattle the potency of a tuberculin is estimated by comparing the size of the reaction elicited by an intradermal inoculation, and comparison to the size of the reactions of a “standard” tuberculin of known potency. To meet the requirements of statistical analysis each tuberculin is used at two dilutions, usually, at normal strength (which for many tuberculin is 1mg/ml) and 20% of normal strength. To determine the potency of batches of bovine PPD, VISAVET will carry out the assay in a level 2 bio-safety farm housing cattle giving positive result to a single intradermal tuberculin test. In summary, in each assay the potency of three routine use batches is estimated by assay against an International Standard bovine tuberculin (CVI, Lelystad) used at dilutions of 1 mg/ml and 0.2 mg/ml. The three bovine PPDs under assay are also used at dilutions of 1 mg/ml and 0.2 mg/ml. The tuberculin preparations will be inoculated into each animal at four sites on each side of the neck according to assay worksheets. The skinfold thickness at the injection sites will be measured and recorded on the worksheets. A statistical analysis of the increase in skinfold thickness at each site will be done to estimate the potencies.

The PPDs and antigens will be also evaluated in the interferon-gamma assay (IFN- γ). This *in vitro* test, also measuring the cellular mediated immunity, is based on the detection of IFN- γ in plasma supernatants from tuberculin-stimulated whole blood culture. Comparative evaluation of different PPDs and antigens will be carried out in blood from infected cattle and goats housed at the research farm and/or field trials. The true infection status to determine sensitivity and specificity will be determined by post-mortem studies (presence/absence of macroscopic lesions and samples will be collected for culture of mycobacteria).

Moreover, studies will be performed to determine the influence of the different tuberculin concentrations in the IFN- γ results. The objective of this study is to evaluate and alternative system to reduce animal experiments and improve animal welfare. In this case, blood samples stimulated with tuberculin at different concentrations will be tested by IFN- γ assay and the results will be compared with the skin test.

At the end of 2011 a workshop will be organized to present the obtained data with the different tuberculins used in the EU to the European Commission and the National Reference Laboratories (see chapter 1.8).

The duration of this task will be yearly to be continued during the following years.

1.2. Diagnosis of tuberculosis in camelids.

The Commission has suggested the inclusion of a new activity for the period 2011 related to TB in camelids particularly regarding reliability, accuracy, sensitivity and predictive value of negative results of the available diagnostic tests with special attention to the tuberculin test.

Due to the interest showed by the Commission in this subject, the EU-RL for Bovine Tuberculosis has prepared an exhaustive report describing the state of the art of diagnosis of tuberculosis in camelids (see Appendix). This assessment of the tuberculosis diagnostic tests for detection of infected camelids could be published as a review to be available for the Commission, all Member States and the Scientific Community.

The review of the published studies on tuberculosis diagnosis reveals the following aspects regarding diagnosis of tuberculosis in camelids:

- There is a lack of data on performance of diagnostic tests in naturally infected and non-infected camelids (with known infection status). When data from known-infected animals sample sizes are available, sample size is usually very limited, and thus estimation of reliability of tests for detection of infected animals has to deal with a large uncertainty.
- Most of the studies report negative test results from animals from which no follow-up is available. However, it is not possible to exclude the possibility that some of them could be truly infected.

- Tests performed on experimentally infected animals show better sensitivity than when applied on naturally infected animals. This suggests that challenge of camelids might not represent a real natural infection, and therefore interpretation of data from experimental infections should be done carefully.
- Usually few animals react in the skin tests after infection is detected in a herd. This can suggest a lack of sensitivity of the tests but also a low transmission rate of tuberculosis between camelids (several studies report only one or two clinically infected animals in herds that are not followed by more reactors in consecutive tests).
- Very few data on performance of different diagnostic tests on Old World camelids exists, even with skin tests. Available evidence suggests that tuberculin test should be performed on the axillary site and read five days after inoculation, but studies on larger number of animals would be necessary to corroborate this hypothesis.
- Serological tests seem to offer promising results for tuberculosis diagnosis. However, its usefulness for early diagnosis and the lack of false positive reactions in animals exposed/infected with other mycobacteria remains to be fully determined.

After analysis all the available information it is clear that there is a lack of knowledge on many aspects of tuberculosis diagnosis in camelids, and consequently a need to improve available diagnostic tests to help the control and eradication of the disease in these animal species (and to prevent transmission to domestic livestock). In this sense, the EU-RL for Bovine Tuberculosis considers that actions should be taken to determine the sensitivity and specificity of available diagnostic tests under two different conditions:

- For estimation of tests' sensitivity, animals from infected herds should be tested and then slaughtered and subjected to post-mortem analysis (detection of macroscopical lesions and, when possible, isolation of tuberculosis-causative agent).
- For specificity analysis, animals from herds known to be free of tuberculosis should be tested. If abnormal responses to any test are observed, further analysis of reactors should be considered to determine the cause of potential unspecific reactions. If possible, analysis of tuberculosis free-herds under different environmental conditions (especially regarding exposition to other mycobacteria, such as *M. avium* subsp. *paratuberculosis* or environmental mycobacteria) should be contemplated to evaluate their importance as cross-reacting agents.

The diagnostic tests included in the evaluation should be the most widely used techniques: SIT and SCIT tests and serological tests (ELISA, RT and MAPIA).

Due to economical and logistical difficulties to perform these studies in Europe, it is considered that a more realistic approach could be its development under natural conditions in a South American country in New World Camelids. Therefore, activities included in 2011 programme will be aimed at studying reliability of diagnostic techniques in infected and non-infected alpacas' herds in Peru.

Phase one of the trial will be the identification of these tuberculosis-infected and tuberculosis-free herds. For this purpose, epidemiological data regarding presence of compatible symptoms, previous sanitary history of the herds and contacts with potentially infected animals will be collected with the collaboration of Peruvian veterinarians. In the case of tuberculosis-infected herds, confirmation of the presence of the disease will be carried out by post-mortem analysis of animals sent to the slaughterhouse.

For sensitivity analysis, all tested animals will have to be culled to determine their true infectious status using gold-standard techniques (post-mortem analysis for detection of macroscopical lesions compatible with tuberculosis and/or bacteriology). Therefore, the goal will be to purchase animals from two-three herds up to a number of 100 animals. These animals will be tested with the techniques mentioned above and will be then slaughtered in local abattoirs to do the post-mortem analysis and collection of samples. Depending on the availability of laboratories capable of performing *M. tuberculosis complex* isolation, samples will be processed in a Peruvian laboratory or submitted to the facilities of the European Union Reference Laboratory for Bovine Tuberculosis for etiological diagnosis. Once the true status of tested animals is determined, ability of the different diagnostic tests applied to detect infected animals will be calculated, as well as individual factors that could potentially affect diagnostic outcome.

For specificity analysis, a larger number of animals will be tested, as in this situation there is no need of culling all animals (as long as the epidemiological evidence to consider the selected herds tuberculosis-free is solid enough). Therefore, up to 300 animals from several herds (ideally subjected to different potentially confounding factors, such as exposition to environmental cross-reacting bacteria or infection with other mycobacteria such as *M. avium* subsp. *paratuberculosis*) will be tested using the

different diagnostic tests. A number of animals (up to a 20% depending also on the reactions observed) will be culled to i) assure the TB-free status of the herd and ii) determine the possible causes of the non-specific reactions. These animals would be subjected to post-mortem analysis and bacteriology analysis to detect both mycobacterial or other bacterial infections.

In total, a number of not more than 160 alpacas (in both sensitivity and specificity trials) should be purchased in order to ascertain their true infectious status regarding tuberculosis infection.

The duration of this task will be yearly to be continued during the following years.

1.3. Determine the reliability of the tuberculin test in cattle infected with *M. caprae*.

The issue “*Mycobacterium caprae*: situation in EU and legislation” was addressed during the First Meeting of the European Union Reference Laboratory for Bovine Tuberculosis (Madrid 4th-5th December, 2008). During this meeting a great concern was shown regarding the legal problem associated to the identification of the pathogen in a herd because taxonomically is now considered to be independent from *M. bovis*. During the open discussion about the situation in the different countries, representatives from several countries (Austria, Germany, Hungary, Italy, Slovenia and Spain reported isolation from cattle and/or wildlife and specific problems regarding reporting system (ie. different names use for reporting). In summary, it was concluded that *M. caprae* is a pathogen that poses a serious problem for Animal Health not only for goats but also to other domestic and wild animal species, and it has been demonstrated to be a human pathogen. Therefore, relevant legislation should be adapted to address the infection, and notification and management of outbreaks caused by this pathogen should be performed as with *M. bovis*. The EURL for Bovine Tuberculosis elaborated a report with the current knowledge on this pathogen summarizing the open discussion in the first meeting in order to recommend further actions.

As a continuation of this work on *M. caprae*, the EU-RL for the next period 2011 will determine the reliability of the skin test in cattle infected with *M. caprae*. To optimize the available resources the first approach will be to get in contact with all the countries that confirmed to have cattle infected with *M. caprae* to request information regarding tuberculin test readings and post-mortem results (lesions/culture) to perform

retrospective studies determining the sensitivity of the test. Another approach to confirm the feasibility of this diagnostic test in cattle infected with *M. caprae* would be to confirm the eradication of an outbreak due to *M. caprae* in a cattle herd using the skin test. At last and once all the results have been analyzed, a field study to determine the sensitivity in a known cattle herd infected with *M. caprae* will be considered.

The duration of this task will be yearly to be continued during the following years depending on the preliminary results.

1.4. Supply spoligotyping membranes to NRL.

The spoligotyping membrane, essential to perform the spoligotyping protocol, can be obtained commercially by only one supplier (Ocimum Biosolutions Ltd., India), although in the recent years problems regarding quality controls have been encountered. To avoid this problem, the EU-RL for bovine tuberculosis is working in the development of home-made spoligotyping membranes to be able to fulfill all the quality standards required. Therefore, a new task for the next period is to supply this home-made spoligotyping membrane to all the NRL that would like to implement this technique in their own laboratories to make sure the high-quality of the membrane and that the results obtained are reliable. VISAVET has all the equipment necessary to develop and test this protocol and will submit the membranes upon request of the different laboratories. The duration of this task will be yearly to be continued during the following years.

A main activity of the EU-RL for bovine tuberculosis is the organisation of periodic ring trials for standardization of techniques and setting up or harmonisation of different protocols at the National Reference Laboratories. Therefore during the next period a ring trial for typing members of the *M. tuberculosis* complex with the home-made supplied membranes will be organized to test the performance of the test in the different NRLs. The duration of this task will be yearly.

1.5. Manual review of the protocols available in the NRLs.

As described in the technical report 2009 regarding harmonisation of protocols, the different protocols applied throughout the MSs were described in the workshop based on the results of the three questionnaires (culture and identification, molecular typing and gamma-interferon test) sent to all MSs. The need to homogenize the protocols was

also discussed in the meeting although some MSs were reluctant to change protocols. To show the difficulty in harmonisation of the protocols a manual review of the different protocols used in the NRLs will be done and submitted to the Commission and all Member States. The duration of this task will be yearly.

1.6. Comparative tests.

A main activity of the EU-RL for bovine tuberculosis will be the organisation of periodic ring trials for standardization of techniques and setting up or harmonisation of different protocols at the National Reference Laboratories. In the first workshop organized in 2008 the priorities for the ring trials were discussed, being evaluation of performance of IFN- γ ELISAs in the laboratories and direct diagnosis by PCR the main options preferred. Therefore during the next period a ring trial for direct PCR from clinical and/or spiked tissue samples will be organized. As mentioned previously, a comparative test will be also performed with the home-made spoligotyping membrane sent to NRLs.

The duration of this task will be yearly to be continued during the following years.

1.7. Missions.

For 2011 there are two missions programmed although the EU-RL for bovine tuberculosis is available for any other missions proposed by the Commission.

The Spanish National Reference Laboratory for bovine tuberculosis in Santa Fe (Granada) has organized two collaborative ring trials for IFN- γ analysis during 2008 and 2009 with the participation of more than 30 laboratories all over Spain. Due to its experience in organizing this type of ring trials and since the EU-RL for bovine tuberculosis will have to organize a ring trial for IFN- γ diagnosis, a visit to the NRL is planned for 2011.

Moreover, a visit to South-America (Peru) is programmed to organize the field experience regarding diagnosis of tuberculosis in camelids, as well as to establish contact with the laboratories that will undertake the diagnosis tests (skin test, serological tests, post-mortem analysis and bacteriological culture).

The duration of this task will be yearly to be continued during the following years.

1.8. Workshop.

A workshop will be organized at the end of 2011. The main topic for this workshop will be to present the results obtained in the potency test studies performed in all tuberculins available in the European Union. Participants will include a member from each National Reference Laboratories in the Member States and delegates from the Commission. If approved from the Commission members from the Scientific Community will be invited to actively participate in the discussion. A report according to rules defined by the Commission would be prepared.

The duration of this task will be monthly (approximately November-December 2011).

2. Other activities of the EU-RL for Bovine Tuberculosis for 2011.

2.1. Isolation and identification of *Mycobacterium* spp.

Bacteriology diagnosis of *Mycobacterium* spp. from clinical samples from domestic and wild animals will be available for Member States. Bacterial culture remains the “gold standard” method for confirmation of infection. In addition, microscopic examination is useful for a presumptive confirmation of acid fast bacilli.

Culture procedure will be carried out in bio-safety level 3 laboratories using samples from domestic animals and other species, including wildlife. Both solid media and liquid media will be used. Identification of isolates will be carried out based on DNA extraction and subsequent amplification by Polymerase Chain Reaction (PCR) targeting genus, species or complex-specific sequences. Sequencing may be used to characterise key genes.

The duration of this task will be yearly to be continued during the following years.

2.2. Typing *Mycobacterium* spp. strains.

Molecular epidemiology is an integration of conventional epidemiology with molecular techniques to track specific strains of pathogens in order to understand the distribution of disease in populations. Molecular typing of isolates has become a valuable tool in the study of tuberculosis epidemiology allowing investigators to detect outbreaks and achieve better knowledge of transmission and increased incidence of infection.

A wide variety of DNA-fingerprinting techniques have been developed to differentiate the *M. tuberculosis* complex isolates and epidemiological purposes. PCR-based fingerprinting techniques have been implemented in most laboratories such as the direct variable repeat (DVR)-spoligotyping and the mycobacterial interspersed repetitive units (MIRU)-variable number tandem repeat (VNTR) typing. Some laboratories only perform the DVR-spoligotyping routinely but in certain epidemiological situations the combination with VNTR analysis offers a better discrimination that could be useful in some epidemiological studies.

Mycobacterium spp. submitted by National Reference Laboratories will be characterised by molecular tools that would be updated according to scientific literature and international acceptance. Initial choice would be DVR-spoligotyping and additional MIRU/VNTR analysis. Spoligotyping numbering will follow the code number from the website that hosts the spoligotype database of *Mycobacterium bovis* and *Mycobacterium caprae* strains (www.mbovis.org). MIRU/VNTR analysis will follow the protocol agreed at the VENoMYC network.

The duration of this task will be yearly to be continued during the following years.

2.3. A *Mycobacterium* spp. culture collection.

A collection of *Mycobacterium* spp. causing tuberculosis in animals is being organized and maintained. This collection is mainly composed of *M. bovis*, *M. caprae* and *M. tuberculosis* isolates from domestic and wild animals, but include as well other important veterinary pathogens such as *Mycobacterium avium* subsp. *paratuberculosis*, *M. a.* subsp. *hominissuis* and atypical mycobacteria. These isolates will be used for evaluation of bacteriology-based diagnosis and molecular characterisation. Isolates will be supplied to National Reference Laboratory upon request.

The duration of this task will be yearly to be continued during the following years.

2.4. Preparation and control of reference reagents.

The EU-RL will prepare and control the reference reagents in order to standardise the protocols used in the different countries and to validate the ring trials. Also, reagents submitted by National Reference Laboratories will be evaluated. Substances, reagents and other biological materials available or prepared at VISAVET will also be available to National Reference Laboratories for harmonisation of protocols; i.e. *Mycobacterium*

bovis BCG (Danish) CCUG 27863 (internal reference code VV-E-457), *Mycobacterium avium* subsp. *avium* CCUG 20992 (ATCC 25291) (VV-E-480), *Mycobacterium tuberculosis* CCUG 37357 (H37Rv) (VV-E-481), *Mycobacterium avium* subsp. *paratuberculosis* CIP 103963 (VV-E-523), recombinant Bovine IFN-gamma RYD-2300-BG-025 (08/0737) or bovine plasma in lithium heparin IBN-N-12 (08/0868).

The duration of this task will be yearly to be continued during the following years.

2.5. Keeping abreast of developments.

Staff of the EU-RL for bovine tuberculosis will keep abreast of developments in surveillance, epidemiology and prevention of tuberculosis throughout the world. To fulfil this commitment, the members of the Laboratory will get information through different ways (scientific papers in national or international journals, attendance to congresses, and workshops, specific training courses, reports from experts, legislation, etc.) and also through active participation in research projects. The relevant information will be distributed to the Commission and the National Reference Laboratories.

The duration of this task will be yearly to be continued during the following years.

2.6. Dissemination.

The existence, role and tasks of the EU-RL for Bovine Tuberculosis will be disseminated via national and international routes to several levels. The information, previous authorization from the Commission, will be disseminated mainly through:

- Presentations at international and national congresses or conferences, publication in international and national journals;
- World Wide Web page which will contain basic updated information.

The duration of this task will be yearly to be continued during the following years.

2.7. Training of personnel.

Training of experts from the Member States and from third countries (when appropriate) will be performed by organisation of short courses or by individual training. These short visits will be open to all National Reference Laboratories to allow the establishment of new protocols and techniques in their laboratory of origin. Afterwards, the trainees will be requested to submit a brief report.

The duration of this task will be yearly to be continued during the following years.

2.8. Technical assistance to the Commission.

The staff of the EU-RL for bovine tuberculosis will be accessible to provide technical assistance to the Commission and upon its request this will be extended also to its Institutions. The staff could also provide support to Member States on specific issues regarding eradication programs. A contact with Public Health Institutions would be established in order to increase awareness of the zoonoses. Moreover, the Director of the EU-RL will participate in the bovine tuberculosis subgroup of the Task Force.

2.9. Research activities.

The EU-RL for bovine tuberculosis will maintain its active research directed towards the improved control and eradication of bovine tuberculosis through: a) Collaboration with National Reference Laboratories, i.e. relevant problems associated to local farming practices on the epidemiology of the infection in the Member States (livestock breeding systems and specific role of wildlife) and impact on detection of infection in animals; and carrying out validation trials; and b) Analysis of the information collected and preparation of reports associated to the activities of the EU-RL.

The EU-RL for bovine tuberculosis will maintain research activities at international level participating in the EU project TB-STEP “Strategies for the eradication of bovine tuberculosis” FP7-KBBE-2007-1, co-ordinated by Dr. Lucas Domínguez. The consortium is made up of 12 partners from eight countries which research on eight workpackages devoted to improved tools and to develop strategies for the eradication of bovine tuberculosis in areas where the disease is present in both domestic and wildlife populations. It includes: 1) vaccination of bovine animals and wildlife, (2) control of populations to reach numbers compatible with animal welfare and strategies to limit the contact between domestic and wild species, and (3) the development of improved diagnostic tools for detection of infected animals.

Moreover, VISAVET is taking part in a new proposal for an European Commission COST Action project titled “FoodMyc” that will be focused on the dissemination of knowledge among scientists working on mycobacteria research.

The duration of this task will be yearly to be continued during the following years.

Appendix

Assessment of tuberculosis diagnostic tests for detection of infected camelids.

Assessment of tuberculosis diagnostic tests for detection of infected camelids.

Animal tuberculosis is caused by members of the *Mycobacterium tuberculosis* complex; among them, the bacterial species most frequently involved in animal infections are *M. bovis* and, to a lesser extent, *M. caprae*. Both of them can infect a wide number of animal species, especially *M. bovis*, which has been shown to have an extraordinary host range (1). This host range includes both domestic and wild animals, and can complicate the fight against animal tuberculosis in those species subjected to eradication/control programs (mainly cattle), as many animal species can act as reservoirs of infection and therefore as potential sources of tuberculosis for livestock and humans.

Among susceptible species to the tuberculosis causative agents, New World camelids (NWC) or South American camelids have gained greater importance in Europe in the last decades, due to the growing number of animals (mainly llamas – *Lama glama* – and alpacas – *Lama pacos*) being imported to several European countries to serve as pets, pack animals or productive purposes (2). Moreover, Old World camelids (Bactrian camel – *Camelus bactrianus* – and dromedary – *Camelus dromedaries*) can be also infected by *M. tuberculosis* complex members (3,4,5). Tuberculosis has been reported to occur more often when animals are managed under intensive conditions and/or close to cattle herds (2,4), with little incidence of infection in NWC in their natural habitat, maybe indicating a lower susceptibility to mycobacterial infections under natural conditions (6). However, a higher susceptibility to *M. bovis* in llamas has been also suggested (7). Transmission between camelids [alpacas (8) and llamas (6)] has been also suspected in European herds.

Diagnosis of tuberculosis infection in these species is challenging, as common diagnostic tests have proven to lack both sensitivity and specificity (9). Detection of disease is frequently made due to clinical diagnosis, although symptoms are frequently non-specific (wasting, respiratory distress and sometimes sudden death). Evaluation of the reliability of the available techniques is seriously impaired by the lack of systematic studies specifically aimed at determining their usefulness on animals of known status infections. A number of reports describing the application of tests based on detection of the cellular (tuberculin test) and humoral (serological tests) immune response on infected hosts have been published. However, the lack of knowledge of the true status of the majority of tested animals allows very limited interpretation of data. Table 1 and 2 shows the performance of cellular and humoral immune response-based tests in **animals with a known infection status**. Results have been included regardless sample size, although 95% confidence intervals for sensitivity and specificity estimates have been included in an attempt to reflect the uncertainty associated with small numbers of animals studied. For specificity estimation purposes only results from animals with no known exposition to *M.*

tuberculosis complex members have been included (thus all animals from herds where infected individuals were detected have been excluded from the analysis).

The diagnostic techniques aimed at the detection of the immune response of infected animals have been the basis of tuberculosis control and eradication programs in domestic ruminants, and thus have been the most widely investigated tests. In the present review of the literature on diagnosis of tuberculosis in camelids, some results of both Single Intradermal Tuberculin (SIT) and Single Comparative Intradermal Tuberculin (SCIT) tests are presented; in an attempt to minimize false positive reactions due to sensitization to environmental mycobacteria, SCIT test have been used more frequently. Several sites have been assayed to maximize the sensitivity of the test in camelids (mainly neck and axillary sites, but also under the tail). Due to the thick and resilient nature of the skin of the neck, the axillary site is usually preferred (10,11). Different reading times have been also evaluated to measure the skin fold difference, and maximum values have been obtained 72-96 hours post-inoculation of the antigens in NWC (7), although apparently there is limited information on this subject. The only comparative assay in Old World camelids performed on infected animals (12) showed higher measures five days post-inoculation, although it was only performed on two animals.

Although humoral immune response is known to occur primarily in late stages of infection (and therefore its detection has little application for early detection purposes) a number of in-house and commercial techniques have been applied for tuberculosis diagnosis in camelids. Among them, in-house ELISAs for specific detection of antibodies against crude extracts of mycobacteria or recombinant antigens have been used with different results. Other approaches, such as the multiantigen print immunoassay (MAPIA), capable of detecting antibodies against a cocktail of selected antigens applied to nitrocellulose membranes (13) and a lateral-flow-based rapid test (RT) detecting antibodies against a set of recombinant tuberculosis antigens and giving a result in 15-20 minutes (14,15) have shown their usefulness as diagnostic tests, alone or in combination with skin testing (see below). However, due to the lack of light chains in camelid antibodies (16,17) a limited flexibility of the immune complex formed by sample antibody and antigen-coated latex particles could difficult their detection using the RT thus causing a decrease in test specificity in certain cases (11).

Other techniques seldom used for tuberculosis diagnosis in camelids include Fluorescence Polarization Assay (FPA) (18) and detection of gamma-interferon (IFN- γ) assay (19).

Table 1. Sensitivity and specificity of skin tests performed on camelids with known infection status

Test ^a	Animal species tested (n)	True status (bacterial species)	Sensitivity (95% CI)	Specificity (95% CI)	Comments	Reference
SIT test	Alpaca (16)	Experimentally infected (<i>M. bovis</i>)	100 (79.4-100)	ND	No data on site of inoculation and interpretation criteria	de la Rúa Domenech, 2005 (in Cousins and Florisson, 2005)
	Llama (5)	Experimentally infected (<i>M. bovis</i>)	80 (28.4-99.5)	ND	Axillary site, day 80 post-challenge, readings at 96 hours post-inoculation	Stevens <i>et al.</i> (1998)
	Llama (3)	Experimentally infected (<i>M. bovis</i>)	100 (29.2-100)	ND	Axillary site, day 143 post-challenge, readings at 96 hours post-inoculation	Stevens <i>et al.</i> (1998)
	Dromedary (2)	Naturally infected (<i>M. bovis</i>)	100 (15.8-100)	ND	Axillary site, reading 5 days post-inoculation, standard interpretation	Wernery <i>et al.</i> (2007)
	Alpaca (12)	Non-infected	ND	100 (73.5-100)	No data on site of inoculation and interpretation criteria	de la Rúa Domenech, 2005 (in Cousins and Florisson, 2005)
	Llama (2)	Non-infected	ND	100 (15.8-100)	Axillary site, performed twice in both animals	Stevens <i>et al.</i> (1998)
SCIT test	Alpaca (2)	Naturally infected (<i>M. bovis</i>)	0 (0-84.2)	ND	Test performed in the month before the onset of clinical signs.	García-Bocanegra <i>et al.</i> (2010)
	Alpaca (21)	Experimentally infected (<i>M. bovis</i>)	76.2 (52.8-91.8)	ND	No data on site of inoculation and interpretation criteria	de la Rúa Domenech, 2005 (in Cousins and Florisson, 2005)
	Llama (14)	Naturally infected (<i>M. bovis</i>)	14.3 (1.8-42.8)	ND	Axillary site, no data on interpretation criteria	Dean <i>et al.</i> (2009)
	Llama (7)	Naturally infected (<i>M. microti</i>)	0 (0-41)	ND	Axillary site, standard interpretation	Lyashchenko <i>et al.</i> (2007)
	Llama (24)	Experimentally infected (<i>M. bovis</i>)	87.5 (67.6-97.3)	ND	No data on site of inoculation and interpretation criteria	Stuart, 2005 (cited in Cousins and Florisson, 2005)
	Dromedary (2)	Naturally infected (<i>M. bovis</i>)	100 (15.8-100)	ND	Axillary site, day 143 post-challenge, readings at 96 hours post-inoculation	Wernery <i>et al.</i> (2007)
	Alpaca (12)	Non-infected	ND	100 (73.5-100)	No data on site of inoculation and interpretation criteria	de la Rúa Domenech, 2005 (in Cousins and Florisson, 2005)
	Llama (12)	Non infected	ND	100 (73.5-100)	No data on site of inoculation and interpretation criteria	Stuart, 2005 (in Cousins and Florisson, 2005)

^a**SIT**: Single Intradermal Tuberculin; **SCIT**: Single Comparative Intradermal Tuberculin.

Table 2. Sensitivity and specificity of serological tests performed in camelids with known infectious status

Test ^a	Animal species tested (n)	True status (bacterial species)	Sensitivity (95% CI)	Specificity (95% CI)	Comments	Reference
MAPIA	Llama (14)	Naturally infected (<i>M. bovis</i>)	100 (76.8-100)	ND	MPB83 was the serodominant antigen	Dean <i>et al.</i> (2009)
	Llama (7) and Alpaca (1)	Naturally infected (<i>M. microti</i>)	87.5 (47.3-99.7)	ND	MPB83 was the serodominant antigen	Lyashchenko <i>et al.</i> (2007)
	Alpaca (13)	Non-infected	ND	100 (75.3-100)		Lyashchenko <i>et al.</i> (2007)
	Llama (33)	Non-infected	ND	100 (89.4-100)		Lyashchenko <i>et al.</i> (2007)
	Dromedary (3)	Naturally infected (<i>M. bovis</i>)	100 (29.2-100)	ND		Wernery <i>et al.</i> (2007)
RT	Llama (14)	Naturally infected (<i>M. bovis</i>)	64.3 (35.1-87.2)	ND		Dean <i>et al.</i> (2009)
	Llama (6)	Naturally infected (<i>M. bovis</i>)	100 (54.1-100)	ND	Anamnestic test (three weeks after SCIT test)	Dean <i>et al.</i> (2009)
	Llama (7) and Alpaca (1)	Naturally infected (<i>M. microti</i>)	62.5 (24.5-91.5)	ND		Lyashchenko <i>et al.</i> (2007)
	Alpaca (13)	Non-infected	ND	92.3 (64-99.8)		Lyashchenko <i>et al.</i> (2007)
	Llama (33)	Non-infected	ND	87.9 (71.8-96.6)	Animals from two herds; all false positive reactors came from the same herd	Lyashchenko <i>et al.</i> (2007)
	Dromedary (3)	Naturally infected (<i>M. bovis</i>)	100 (29.2-100)	ND		Wernery <i>et al.</i> (2007)

^a**MAPIA**: Multiantigen print immunoassay; **RT**: lateral-flow-based rapid test.

Studies performed in new world camelids.

Stevens and collaborators (7) challenged six llamas with different doses of *M. bovis* to measure their immune responses using SIT test (performed in the axillary site) and an ELISA aimed at the detection of specific antibodies against *M. bovis*, *M. avium* and *M. avium* subsp. *paratuberculosis* antigens at different times after the infection. Skin tests were recorded on five animals 80 days after infection (4/5 reactors with skin increase above 2 mm.) and on three animals 143 days post-infection (3/3 positive animals with reactions above 2 mm.) due to premature death of some of the animals due to the tuberculosis infection. Reactions were maximum 96 hours post-bovine PPD inoculation. Two non-infected control animals showed no reactions at all times. Results of serological analysis using *M. bovis* antigens were consistently positive in most animals from day 83 post-infection onwards, although one animal reacted as soon as 34 days after the challenge. However, extrapolation of tests performance to what could be expected under natural conditions is difficult due to the heavy infection that was induced in all challenged animals (with three out of six infected animals dying before the completion of the experiment) and the antibiotic treatment given to all animals but one.

SIT test was capable to detect all 16 experimentally infected alpacas 100 days after inoculation with *M. bovis*, and correctly classified 12 non-infected animals; SCIT detected 16 out of the 21 alpacas challenged with *M. bovis*, and also showed a perfect specificity when performed on 12 non-infected alpacas [R. de la Rúa-Domenech, personal communication; cited in (1)]. A similar experiment was performed on 24 llamas experimentally infected with *M. bovis* and 12 uninfected controls: SCIT test detected 21 of the challenged animals, and all controls tested negative. An ELISA detecting antibodies against bovine and avian PPDs detected all 24 infected animals, but no results on specificity of the assay are provided [F. Stuart, personal communication, cited in (1)].

These reasonable figures for sensitivity and specificity of tuberculosis diagnostic tests contrast with performance of tests when naturally infected animals are analyzed. As an example of lack of sensitivity of SCIT test to detect all infected animals in a herd under natural conditions, Twomey and collaborators (20) reported the results obtained in a llama herd in United Kingdom where tuberculosis infection was detected: after finding the first positive animal (euthanized due to poor body condition) all herd was tested every 90 days using SCIT test. After two rounds with all animals testing negative, a new clinically ill animal was detected and tuberculosis infection was confirmed by isolation of *M. bovis*. Three months later a new SCIT test was performed, with three animals showing positive reactions, although no data on the infection status of these animals was provided by the authors. Animals leaving the herd in the 12 months before the onset of the outbreak were traced back and skin tested, but all yielded negative

results. In this herd the serological RT test was performed after the second SCIT test, and 19 adults and two crias were culled based on serological results or epidemiological suspicions, from which tuberculosis infection was confirmed in four adults. A more detailed report on performance of diagnostic techniques (SCIT test, and RT and MAPIA serological tests) on a subset of alpacas from this herd was published by Dean and collaborators (21). Skin test was only able to detect two out of 14 animals with macroscopical lesions compatible with tuberculosis (and positive culture of *M. bovis* in 12 cases), while MAPIA detected all 14 individuals. RT performed before the tuberculin test detected nine out of the 14 infected animals; however the analysis was repeated in six animals (four positive and two negative in the previous round) after the skin test and all tested positive suggesting a potential increase in the sensitivity.

The risk of introduction of tuberculosis due to *M. bovis* associated to animal movement was demonstrated in three Alpaca herds (8). Again in these cases, tuberculosis infected animals were detected due to clinical symptoms, but once diseased animals were removed from the herd SCIT test was performed in all three herds and no reactors were found. Again lack of information of the true status of negative SCIT test individuals make difficult to ascertain if all were “true negative” animals.

A lack of sensitivity of SCIT test in alpacas infected with *M. bovis* was also reported by Garcia-Bocanegra and collaborators (19), as two infected herds (of 32 and four animals) were analyzed (one month before the clinical diagnosis of one infected animal in one case, and one week after observing clinical symptoms in another animal in the second herd) and all animals were negative. The usefulness of the gamma-interferon (IFN- γ) detection assay designed for livestock (Bovigam, Prionics AG, Switzerland) was evaluated, but no positive results were obtained.

Other members of *M. tuberculosis* complex as *M. microti* (6) can also cause tuberculosis in NWC, thus provoking the same diagnostic problem. In this sense, SCIT test performed on two llama herds from Switzerland after one *M. microti* infected animal was detected in each one yielded negative results in two rounds at 6-week intervals, what was interpreted as a signal that no more infected animals remained in both herds (6). Interestingly, one of the infected animals came from South America in a group where six out of 83 llamas showed inconclusive reactions in the tuberculin skin test, maybe indicating an early exposition to the tuberculosis causative agent. The second infected animal was an offspring of another female from this same group, thus revealing a potential epidemiological link.

Serological diagnostic test have been also assayed in alpacas and llamas infected or exposed to *M. microti*, showing promising results (11). RT test detected five out of eight confirmed or strongly suspected *M. microti* infected animals (seven llamas and one alpaca) while all animals

reacted in the MAPIA test. Estimated specificity of both tests was high also (89.9 and 97.5% for RT and MAPIA tests respectively), although some exposed animals were included among the true negative individuals due to the lack of symptoms (if these animals are excluded, specificity values would be 89.9 and 100%). Interestingly, false positive responses in non-exposed animals to the RT test varied depending on the herd of origin (from 0 to 20% of non-specific responses detected, thus indicating potential differential exposure to cross-reacting antigens). No significant differences were observed between the responses obtained from alpaca or llama samples.

Studies performed on old world camelids.

There are few detailed reports on the effectiveness of diagnostic tests in Old World camelids, and especially cases of tuberculosis in Bactrian camel seem rare. High levels (10-20%) of non-specific reactions were reported (22) after inoculation of avian and bovine antigens in non-infected Australian dromedaries (as demonstrated by the absence of compatible lesions). Another study using SCIT test in the neck of dromedaries in Kenya (23) reported comparable percentages of reactors to bovine PPD (22%) and avian PPD (34%) from 41 analyzed animals; only two individuals had a larger response to bovine tuberculin than to avian tuberculin. One animal was slaughtered and lesions compatible with tuberculosis (fibrosis and mineralization) were observed, although no isolation of *Mycobacteria* spp. was achieved, and therefore the true infection status of animals could not be determined.

An infection due to *M. caprae* in one dromedary was reported in the context of a larger outbreak affecting also bison in a zoological garden in Slovenia (5). This infected animal was not skin tested, but another dromedary kept in the same pen reacted in the SCIT test and was slaughtered: no lesions were observed, and bacteriology was negative, thus indicating a possible false positive reaction in tuberculin test or a lack of sensitivity in post-mortem analysis.

M. pinnipedii was implicated as the causative agent of another outbreak involving animals from two zoological gardens (four Bactrian camels among them) (3). Isolation of tuberculosis agent could only be achieved in one Bactrian camel, which unfortunately could not be subjected to *ante-mortem* testing. The remaining three animals were analyzed using an in-house detecting antibodies against three antigens: extracts prepared from *M. avium* and *M. bovis* and recombinant MPB70 antigen. Only one animal showed significant titers of antibodies against both *M. avium* and *M. bovis* antigens (and none against MPB70). Although this animal suffered from a severe respiratory disease, lesions found in the post-mortem analysis were not considered pathognomonic of tuberculosis, and nor culture of *M. pinnipedii* neither detection of its DNA could be not achieved, therefore making unclear the significance of high titers against

mycobacterial antigens. The other two Bactrian camels remained healthy and were not subjected to further analysis.

Wernery and collaborators (12) reported the use of both single and comparative skin tests and two serological assays (RT and MAPIA) on a dromedary racing herd (n=57) once one of its members (index case) was diagnosed with tuberculosis. Parallel inoculation of avian and bovine PPDs in the middle of the neck, the axillary site and under the tail (only bovine PPD) demonstrated that higher responses were obtained in two infected animals when the axillary site was used; on the same way, differences on skin thickness in these animals were maximum if the test was read five days after inoculation. Overall results from the 57 animals yielded a total of four positive reactors and four inconclusive reactors to the SIT test; when both avian and bovine PPDs were considered, only two animals were called positive, plus an inconclusive reactor. A high proportion of reactors at avian PPD was recorded (27% of the herd had increases in skin thickness over 2 mm.) which was considered as a signal of exposition to environmental mycobacteria. The whole herd was tested using RT and MAPIA serological tests three times, and only two animals (plus the index case) were found positive by both tests (those that were positive at the SCIT test). These two animals were euthanized and subjected to post-mortem examination although they did not show any clinical symptoms; both were infected with *M. bovis*. No tuberculin reactivity was reported after re-testing the rest of the herd six months later suggesting that non-slaughtered reactors could not have been truly infected, although their true infectious status was not determined. Authors conclude that serological tests were able to detect all proved infected animals, while more non-specific reactions were found in SCIT and especially SIT tests; however, the lack of confirmation of disease on serological-negative animals cannot exclude the presence of tuberculosis infected animals which did not respond to RT and MAPIA tests.

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