In vivo and in vitro diagnosis of Mycobacterium bovis infection

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Summary
The intradermal delayed type hypersensitivity (DTH) skin test, using purified protein derivative from culture of M. bovis or M. avium, is the most frequently used test for diagnosis of tuberculosis or detection of M. bovis infection in cattle. Many improvements have been made to the original tuberculin test, and molecular approaches to identify and clone antigens may lead to improved specificity and sensitivity of DTH skin tests. Recent advances in technology have allowed the development of new in vitro techniques, such as antibody-based, cell-mediated immunity-based and nucleic acid-based diagnostics, which allow more rapid diagnosis than bacteriological culture. The choice of diagnostic technique should consider both the population being investigated (e.g. apparently healthy animals or a herd with a high prevalence of clinical infection) and the aim of the testing (e.g. the screening of healthy animals or confirmation of infection in animals strongly suspected to be infected). Moreover, any evaluation of a diagnostic test must use a carefully selected control population which is representative of the population to be tested in terms of relative proportions of infected and non-infected animals.

Keywords

Introduction
The increasing pressures of modern transportation and trade are creating a requirement for rapid, simple, on-the-farm diagnostic tests for Mycobacterium bovis infection, in addition to mass-scale surveillance testing procedures for the infection in cattle and wildlife populations. During the past century, the tuberculin skin test has been the most frequently used diagnostic test world-wide, approved for use as the primary screening test in most countries for on-the-farm diagnosis of M. bovis infection of cattle. While several improvements have been made to the original intradermal tuberculin test of Robert Koch, the tuberculin skin test has some disadvantages for use in detecting exposed and infected cattle and wildlife, particularly individuals. Comprehensive reviews by Francis et al. (46), Pritchard (106), Wood and Rothel (148) and Martin et al. (81) have focused on advances in technology and enhancing the understanding of the strengths and weaknesses of available diagnostic methods, particularly classical tuberculin tests. As a consequence of identifying some of the disadvantages of the tuberculin skin test, a resurgence of research for the development of improved tuberculosis diagnostic tests has occurred. The aim of this research has been to enhance sensitivity and differential specificity, ease of use, adaptability to automation, computerised interpretation and reporting for individuals, herds and populations and to reduce the cost. The ideal tuberculosis screening test should correctly identify animals infected with M. bovis while avoiding the detection of non-infected animals. Thus, this review will address recent advances that provide alternative rationales and methods to overcome the principal obstacles to improving in vivo and in vitro diagnosis of M. bovis infection and tuberculosis in cattle and wildlife.
Approaches for evaluation of diagnostic tests for *Mycobacterium bovis* infection in cattle

*Mycobacterium bovis* is an obligate pathogen: the presence of *M. bovis* in an animal therefore defines the state of infection. Detection of such animals or populations (herds) is the object of diagnostic tests and is vital to the control and eradication of bovine tuberculosis.

Diagnostic tests may be divided into surrogate and pathognomonic categories. Surrogate tests are those that detect a signal which indirectly indicates *M. bovis* infection, whereas pathognomonic tests are those that directly detect the *M. bovis* organism itself and thus by definition are 100% specific.

The three basic purposes of diagnostic tests are infection discovery, confirmation and exclusion (40). Discovery tests are used for screening apparently healthy livestock populations to detect *M. bovis*-infected animals. A high sensitivity is desired in such assays and modest false-positive rates can be tolerated, since the discovery test is usually followed by a confirmatory test. Confirmatory tests are used when *M. bovis* infection is strongly suspected. Confirmatory tests must have a specificity approaching 100%, but lower sensitivity is acceptable. Exclusionary tests are used to rule out the presence of *M. bovis* infection when suspected. Thus, exclusionary tests must have a sensitivity approaching 100%. Exclusionary tests are usually too expensive for discovery purposes. Some diagnostic tests may be useful for more than one of these purposes.

Evaluation of diagnostic tests for chronic infectious diseases such as bovine tuberculosis is more challenging than for acute diseases. A thorough understanding of the pathobiology of the disease is essential, since different signals of the infection are produced at different phases of the infection. As a result, a particular test may perform better during one phase than during another. Stringent application of case definitions for truly infected and non-infected animals and animal populations is critical to produce a fair and objective evaluation of assay sensitivity and specificity. Equally important is the spectrum of infection severity among the infected animals tested. In addition, the impact of within- and among-herd infection prevalence on the positive and negative predictive values of the tests must be appreciated. For assays that are quantitative, pre-test and post-test likelihood ratios for different levels of test signal can be used to enhance the utility of information provided by diagnostic assays. The fact that tests can be evaluated either for individual animals or the herd level further adds to the challenge.

A detailed description of these clinical epidemiology concepts is beyond the scope of this review. The estimation of the sensitivity and specificity of diagnostic tests for bovine tuberculosis is neither simple nor sufficient. For more thorough explanations of the methods of evaluating diagnostic test performance, the reader is referred to original research publications and reviews by Yerushalmy (150), Feinstein (40), Martin (79), Martin et al. (80, 81) and Sackett (121).

**In vivo diagnosis**

Tuberculin intradermal delayed type hypersensitivity

The tuberculin delayed type hypersensitivity (DTH) skin test, various forms of which exist for bovine and human tuberculosis, may be one of the oldest and the most widely used in *vivo* diagnostic tests. The tuberculin test is a surrogate test that is entirely dependent upon the appropriate cell-mediated immunological response of the host during the various phases of disease pathogenesis. The strains of *M. bovis* used and the production and quality control of tuberculins have been reviewed extensively (56, 132), as well as the evaluation of tuberculins and their injection into livestock species, especially cattle (29, 38, 46, 98, 99). Tuberculins are complex mixtures of soluble antigens (34, 48) produced by mycobacteria (*M. bovis* or *M. avium*) grown as floating cultures in synthetic liquid media, harvested after the mycobacteria are heat killed and removed by filtration followed by heat concentration (old tuberculin) (64) or chemical fractionation (purified protein derivative [PPD]) (125). Purified protein derivative is the antigen of choice for intradermal injection at various doses (46, 52, 98). The PPD from culture of either *M. bovis* (PPD-B) or *M. avium* (PPD-A) is used at a 100 µl volume, either for the caudal fold, the single intradermal cervical or the intradermal comparative cervical test. Increases in skin thickness due to DTH are detected by manual palpation and/or measurement with calipers at 72 h post injection. The caudal fold test for cattle, the single cervical test for deer and the comparative cervical tests require the handling of animals at least twice at a 72 h interval, do not permit repeat testing of the same animal within sixty days, and are more effective when used on a herd rather than individual animal basis.

In cattle vaccinated with bacillus Calmette-Guérin (BCG) or culture-positive for *M. bovis*, PPD-B induces messenger ribonucleic acid (mRNA) expression of gamma interferon (IFNγ), interleukin (IL)-2, IL-4, IL-10 and tumour necrosis factor-alpha (TNF-α) at the injection site at 72 h post injection (94). Publications assessing the diagnostic efficiency of the various skin tests report that the estimated sensitivity ranges from 70% to 90%, while the estimated specificity varies from 75% to 99.8% (29, 46, 66, 81, 118, 143). The caudal fold test was reported to have a sensitivity of 72% with a specificity of...
up to 98.8%; the single intradermal cervical test had a sensitivity of 91.2% with a specificity of 75.5%. The comparative cervical test with PPD-B and PPD-A had a sensitivity of 68.6%-95% with a specificity of 88.8%-99.9% (29, 46, 118, 149), depending upon the circumstances in each country, the interpretation parameters, quality and dose of tuberculins, timing of prior tuberculin test, cross-reacting environmental mycobacteria and other organisms, temporal variations and chronicity of infections in populations, timing of testing relative to calving, and the skill of the applicator.

A direct relationship exists between the predictive value of a positive test and the prevalence of M. bovis infection, while the predictive value of a negative test and the prevalence of infection are inversely related. The rate of false-negatives is influenced by time since exposure to field strain, suppressive/anergic regulation of the immune response, injection of insufficient tuberculin, use of tuberculins of reduced potency, immunosuppression during the early postpartum, desensitisation following tuberculin tests and variability among observers (35, 46, 84). Dietary restriction (36) and the use of oil-based foot and mouth disease vaccines were unrelated to false-negative results (87). Using the example of Martin et al. (81) to further explain the variability of test performance in different cattle populations, applying a tuberculin test with 85% sensitivity and 99% specificity with a 5% prevalence rate, the predictive value of a positive test is 81.7%. However, when the prevalence is less than 1%, more than 50% of the positive results would be expected to be false-positive, thus the apparent prevalence is usually higher than the true prevalence.

To address the issue of false positive tuberculin reactions in humans, individual recombinant proteins and cocktails of cloned recombinant tuberculo-proteins were evaluated for the ability to elicit strong and specific DTH in PPD-positive guinea-pigs or humans (65, 77, 114). Despite protein purification and molecular cloning, no individual M. tuberculosis proteins or cocktails of recombinant proteins were found to eliminate the specificity problems of PPD or to elicit DTH-like responses to PPD. These findings concur with those reviewed elsewhere associated with the genetic diversity of the immune response of outbred cattle populations, especially when highly purified tuberculo-protein antigens are used (148). However, Coler et al. (18) recently cloned and expressed a M. tuberculosis 9 kDa protein, DPPD, which elicits a strong and specific DTH having no cross-reactivity with other pathogenic or environmental mycobacteria, thus providing the first putative specific and sensitive alternative tuberculin reagent for the diagnosis of tuberculosis. The DPPD gene has so far been found in M. tuberculosis and M. bovis-BCG, but not in M. avium, M. kansasii, M. scrofulaceum, M. fortuitum, M. chelonae, M. gordonae, M. terrae, M. smegmatis or M. vaccae (18), which may represent a major advantage for differential diagnosis and reduction of false positives. Thus, modern molecular approaches to identify and clone DTH diagnostic antigens may provide new reagents for detecting M. bovis-infected livestock with equal or greater sensitivity and specificity than current PPD antigens, perhaps even allowing vaccination of animals against tuberculosis while discriminating between M. bovis-infected and vaccinated populations.

**In vitro diagnosis**

**Direct diagnosis via morphology, histochemistry, immunohistochemistry or polymerase chain reaction amplification and/or hybridisation**

Presumptive diagnosis of bovine tuberculosis via DTH response to PPD, clinical history, herd history, clinical findings and gross necropsy findings is significantly reinforced and/or confirmed by histopathological results, histochemical and immunohistochemical staining and especially by in situ hybridisation with M. bovis-specific probes. The gross and histopathological findings and the distribution of tuberculous lesions are directly influenced by the route, dose and virulence of the tubercle bacillus, the susceptibility of the host animal and the time after challenge. The pathogenesis (39, 92) and detailed descriptions of the gross pathology and histopathology of tuberculosis have been extensively described for naturally occurring (6, 13, 26, 39, 90, 109, 137, 144) and experimentally induced tuberculosis (9, 10, 12, 14, 89, 100). However, gross lesion inspections not infrequently fail to detect minor or invisible lesions (particularly in the upper respiratory tract and lymph nodes of the head) in skin-test negative or positive animals that are subsequently found to be M. bovis culture-positive (6, 26, 28, 91, 144), causing concern regarding the efficiency of accurate diagnoses and the associated implications for tuberculosis control programmes.

Thorough gross and histological examination of six pairs of lymph nodes (mediastinal, medial retropharyngeal, bronchial, parotid, prescapular and prefemoral) together with the mesenteric nodes and lungs will detect 95% of cattle with gross tuberculous lesions (6, 26). Following the detection of granulomatous lesions compatible with tuberculosis, the Ziehl-Neelsen (76) or the auramine O (85) acid-fast stains may be applied to impression smears or histosections to identify acid-fast bacilli. If the bacilli are confirmed to be acid-fast, then immunohistochemical staining (14, 54) with antibodies specific to M. tuberculosis complex (not M. bovis-specific) and/or polymerase chain reaction (PCR) (67, 83, 122, 142), using either a 248 base-pair (bp) segment of IS1081 or a 123 bp segment of IS6110, may be performed to specifically identify M. tuberculosis complex organisms in tissues or paraffin-embedded histological sections. (For infection of cattle, when a M. tuberculosis complex organism is identified, the likelihood that it is M. bovis is extremely high.) Immunohistochemical staining of tissue sections had a sensitivity equal to or greater than Ziehl-Neelsen staining (53) on tuberculous tissues and provided a more specific M. bovis.
diagnosis. Tests based on PCR performed on formalin-fixed paraffin-embedded sections with typical tuberculous lesions and acid-fast bacilli were 93% positive (83), whereas PCR-based tests on fresh tissues were 91% positive (142). However, the PCR results were highly specific for \textit{M. tuberculosis} complex mycobacteria and were obtained more rapidly than bacteriological culture (three to four weeks faster). Lastly, \textit{M. bovis} has been detected by a deoxyribonucleic acid (DNA) oligonucleotide probe/PCR assay directly from bovine blood using general A1/B1 primers for spacer regions of the 16S and 23S ribosomal RNA genes specific for the \textit{M. tuberculosis} complex and hybridised with an oligonucleotide probe specific to \textit{M. tuberculosis} complex with a claimed analytical sensitivity of ten organisms in 100 μl of blood (5). Immunohistochemical and PCR-based procedures offer rapid, specific, confirmatory tests for \textit{M. bovis} infection, for instance on sections or tissues with typical tuberculous lesions and acid-fast bacilli, or when herd history, tuberculin test results and necropsy findings are available.

**Bacteriological culture**

The success of primary isolation of \textit{M. bovis} from clinical specimens is influenced by the culture medium employed, the decontamination procedure and incubation conditions (26). Decontamination procedures for the primary tissues may affect the recovery of the mycobacteria significantly, thus these procedures must be followed carefully. Decontamination has been achieved without adverse effects on recovery using 0.075% hexadecylpyridinium chloride (26). Standard bacteriological procedures are well known (101, 134, 137). Most laboratories recommend the use of both an agar-based medium (Middlebrook 7H11) and an egg-based medium (Stonebrink or Löwenstein-Jensen with pyruvate) for primary isolation. Modified Middlebrook 7H11 medium (140), B83 medium (30) and radiometric detection (88) improved the recovery rate and speed of culture when used in conjunction with standard decontamination and concentration procedures. Identification of mycobacterial isolates may be obtained by standard procedures including acid-fast staining, colony morphology, and a panel of biochemical tests (113), such as susceptibility to 2-thiophene carboxylic acid hydrazine (TCH) and isoniazid. These procedures require an additional three to four weeks. The use of monoclonal antibodies (27) or molecular biology techniques reviewed below allow definitive identification of \textit{M. bovis} and DNA fingerprinting of the isolate to be performed in one day.

**Antibody-based diagnostics**

A sensitive and specific serological test to detect antibodies to \textit{M. bovis} antigens in domestic livestock and sylvatic populations exposed to tuberculosis would be very useful to replace or supplement the intradermal test. In general, antibody production by infected individuals is variable, largely undetectable during the early subclinical stages of tuberculosis, and usually occurs during the advanced stages of the disease when cell-mediated immunity tests may be negative due to anergy. The spectrum of purified and complex crude antigens identified and the use of these antigens in a variety of antibody detection procedures, mostly based on the enzyme-linked immunosorbent assay (ELISA), are listed in Table I with comments on the sensitivity and specificity for experimentally and naturally infected cattle (reactivity).

The sensitivities and specificities of virtually all the tuberculosis serological tests, including the indirect and competitive ELISA and Western blot, are relatively poor. This is probably due to the high degree of polymorphism in the antigen recognition, variable kinetics of the antibody response, possible \textit{M. bovis} heterogeneity, and variable rate of disease progression of tuberculosis from animal to animal. Additionally, high rates of false positive serological tests are observed due to extensive cross-reactivity among mycobacterial species. Thus, given the diversity in antigen recognition among cattle, with the possible exception of the memory immunoglobulin (Ig) G, recombinant MPB70 ELISA (73) before and after skin testing, serological tests are best used to complement cell-mediated immunity-based diagnostic tests.

**Cell-mediated immunity-based diagnostics**

Given that the predominant immune response to mycobacterial infections in cattle is cellular rather than humoral, development of in vitro tests to replace or complement the intradermal skin test would greatly facilitate control and eradication programmes. Table II documents studies to develop and evaluate such assays in cattle populations experimentally and naturally infected with \textit{M. bovis}. Although the intradermal skin test can be useful for the detection of infected herds of cattle, the problem of false-positive test results in individual cattle associated with exposure to atypical mycobacteria highlights the need for tests with higher specificity. The developments range from the early lymphocyte blastogenic assays to the detection of specific lymphokines from specific T cells stimulated with crude, purified and recombinant antigens, or synthetic peptides (Table II). Thus, extensive progress has been made in the understanding of the diversity of host cellular immune responses to mycobacterial antigens, yet much remains to be accomplished.

The comparative (PPD-B versus PPD-A) sandwich bovine IFNy ELISA for \textit{M. bovis} developed by Wood et al. (145) and Rothel et al. (120) is the in vitro cell-mediated immune diagnostic test that competes favourably with the skin test for both sensitivity and specificity. The IFNy ELISA has been evaluated world-wide and found to be almost always useful as a complement to skin-testing in most countries and, under some conditions, suitable for use as a primary tuberculosis test. Adaptations and refinements of the sandwich bovine IFNy ELISA concept have allowed investigators to explore the spectrum of bovine host cell-mediated immune responses of T cell subsets to a wide variety of crude and highly purified, recombinant or synthetic antigens with specific epitopes.
### In vitro serological diagnostic tests for the detection of host antibody responses to Mycobacterium bovis antigens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antigen</th>
<th>Nature/antigen preparation</th>
<th>Reactivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovis</td>
<td>PPD</td>
<td>Standard USDA</td>
<td>ELISA: good specificity and reasonable sensitivity</td>
<td>136</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>PPD</td>
<td>Autoclaved TCA precipitate</td>
<td>ELISA: 90% positive in culture positive cattle, 11.1% positive in negative cattle</td>
<td>110</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>PPD</td>
<td>Phenol killed non-autoclaved filtrate</td>
<td>ELISA: less discriminating than PPD</td>
<td>110</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>PPD</td>
<td>Phenol killed non-autoclaved filtrate</td>
<td>ELISA: less discriminating than PPD</td>
<td>110</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>PPD</td>
<td>Autoclaved TCA precipitate</td>
<td>ELISA: sensitivity = 73.95%; specificity = 94.1%</td>
<td>111</td>
</tr>
<tr>
<td>M. bovis WT</td>
<td>Whole cell</td>
<td>Heat killed at 80°C, 120 min sonicate</td>
<td>ELISA: 15.4% positive in negative cattle, up to 39.5% positive in cattle with mycobacteria other than M. bovis</td>
<td>4</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>MPB70</td>
<td>Recombinant MPB70</td>
<td>Western blot: bovine antibody binding to MPB70 detected</td>
<td>107</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>Mycoside B</td>
<td>2-O-methyl-a-L-rhamnopyranosyl</td>
<td>ELISA: bovine anti-M. bovis antibodies highly reactive and specific</td>
<td>17</td>
</tr>
<tr>
<td>M. bovis</td>
<td>PPD</td>
<td>Standard PPD Weybridge</td>
<td>ELISA: only 1/5 M. bovis-infected cattle positive</td>
<td>57</td>
</tr>
<tr>
<td>M. bovis</td>
<td>Phosphatide extract</td>
<td></td>
<td>ELISA: only 3/5 M. bovis-infected cattle positive</td>
<td>57</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>PPD</td>
<td>Standard PPD</td>
<td>ELISA: 39.9% positive in culture positive cattle</td>
<td>112</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>MPB70</td>
<td>Chromatofocused from culture filtrate</td>
<td>ELISA: not specific for M. bovis, 6/19 positive from M. bovis positive cattle</td>
<td>42</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>CF</td>
<td>Culture filtrate</td>
<td>ELISA: 50% positive in M. bovis-infected cattle with low specificity</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>Non-glycosylated MPB70</td>
<td></td>
<td>ELISA: moderately high reactivity. The most highly specific, although not completely species-specific</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>Glycosylated and non-glycosylated MPB70</td>
<td></td>
<td>ELISA: strong reaction with glycosylated MPB70, but with high cross-reactivity</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>MPB64</td>
<td>Chromatofocused from ultrafiltrated, precipitated and dialyzed culture filtrate</td>
<td>ELISA: strong reaction and 50% positive in M. bovis-infected cattle, somewhat species-specific, but more cross-reactive than MPB70</td>
<td>42, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>MPB59</td>
<td>Chromatofocused from ultrafiltrated, precipitated and dialyzed culture filtrate</td>
<td>ELISA: weakest reactivity and unstable</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>P32</td>
<td>Chromatofocused from ultrafiltrated, precipitated and dialyzed culture filtrate</td>
<td>ELISA: weakest reactivity and unstable</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>39 kDa, glycosylated</td>
<td></td>
<td>ELISA: strongest reactions, but with high cross-reactivity</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>HSP65</td>
<td>Cell sonicate</td>
<td>ELISA: strongest reactions, but with high cross-reactivity</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis AN5</td>
<td>HSP70</td>
<td>Culture filtrate</td>
<td>ELISA: strongest reactions, but with high cross-reactivity</td>
<td>43, 44</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>MPB70</td>
<td>Purified from culture fluid</td>
<td>Protein-G-based ELISA: moderate sensitivity, low sensitivity with only 3/9 positive in M. bovis-infected cattle, anti-MPB70 response subject to immune priming by PPD or MPB70</td>
<td>96</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>3G66B</td>
<td>Purified from culture fluid</td>
<td>Protein-G-based ELISA: moderate sensitivity, low specificity</td>
<td>58</td>
</tr>
<tr>
<td>M. bovis WT</td>
<td>Whole cell</td>
<td>Heat killed at 80°C, 120 min sonicate</td>
<td>IgG ELISA: very low specificity (15.5%)</td>
<td>37</td>
</tr>
<tr>
<td>M. bovis</td>
<td>PPD</td>
<td>Standard</td>
<td>ELISA: variable antibody response, enhanced by prior PPD injection</td>
<td>58</td>
</tr>
<tr>
<td>M. bovis</td>
<td>Phosphatide</td>
<td>Saline emulsion of phosphatide extract</td>
<td>ELISA: variable antibody response, enhanced by prior PPD injection</td>
<td>58</td>
</tr>
<tr>
<td>M. bovis</td>
<td>MPB70</td>
<td>Chromatofocused from ultrafiltrated, precipitated and dialyzed culture filtrate</td>
<td>ELISA: sensitivity = 18.1%; specificity = 94.4%</td>
<td>147</td>
</tr>
<tr>
<td>M. bovis</td>
<td>T90/1770 WT</td>
<td>Freeze-thaw-sonicate-SDS-PAGE</td>
<td>Western blot: high sensitivity with 5/10 positive, may be specific for M. bovis</td>
<td>97</td>
</tr>
</tbody>
</table>
Clearly, this research has furthered the understanding of the complexity and diversity of the host immune response. However, specific mycobacterial antigens and peptide epitopes from the antigens studied thus far seem to be of limited practical value in cell-mediated immunity tests, due to the diversity of the immune recognition of these epitopes by outbred cattle (104, 105, 141). A dynamic antigenic repertoire, particularly one containing low molecular weight secreted antigens may improve in vitro cell-mediated immunity assays, particularly during early stages of infection.

These early-stage secreted, more promiscuous antigens potentially offer a new approach for the diagnosis of bovine tuberculosis (103).

**Nucleic acid-based diagnostics**

Since *M. bovis* has the widest host range of the mycobacteria in the *M. tuberculosis* complex, one of the important obstacles to understanding the epidemiology of *M. bovis* transmission among mammalian populations has been the inability to differentiate or type strains based upon phenotypic
Table II

*In vitro* cell-mediated immunity-based diagnostic tests for the detection of host cellular responses to *Mycobacterium* spp. antigens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antigen</th>
<th>Nature/antigen preparation</th>
<th>Reactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Ammonium sulphate precipitation of culture filtrate</td>
<td>Lymphocyte blastogenesis: stimulation index = 18.1 in <em>M. bovis</em> infected cattle, and 1.58 in controls</td>
<td>86, 135</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>Bioassay for bovine IFNγ from PPD-stimulated whole blood detected all cattle experimentally challenged with <em>M. bovis</em></td>
<td>145</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of PPD-stimulated whole blood: highly specific, with results in 24 h and no requirement for testing animals after 3 days, unlike tuberculin test</td>
<td>119</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>MFB70</td>
<td>Chromatofocused from ultrafiltrated, precipitated and dialysed culture filtrate</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of MFB70-stimulated whole blood: significantly reduced sensitivity with only slightly improved specificity</td>
<td>147</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of MFB70-stimulated whole blood: high sensitivity (63.6%) and high specificity (96.2%)</td>
<td>148</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of PPD-stimulated whole blood: high sensitivity (76.8%) to 93.6% and high specificity (95.2%)</td>
<td>146</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of PPD-stimulated whole blood: Optimized by use of heparinized blood stimulated with 20 µg/ml PPD within 8 h of collection</td>
<td>120</td>
</tr>
<tr>
<td><em>M. bovis</em> (1/51/1378)</td>
<td>MBSE and PFB</td>
<td>Ultrafiltered, sonicate Standard PPD</td>
<td>Lymphocyte blastogenesis: stimulation index is high for both MBSE and PPD for 270 days post infection with <em>M. bovis</em></td>
<td>103</td>
</tr>
<tr>
<td><em>M. bovis</em> (1/51/1378), <em>M. avium</em></td>
<td>MBP70-88-105, 144-163 residues, 19 kDa 1-16, 67-84 residues; MBSE and PFB</td>
<td>Synthetic peptides, PPD-B, PPD-A</td>
<td>Lymphocyte blastogenesis stimulation indices (&gt;2.0 = positive); MBP70-88-105 residues = 0.59-21.1, 8/14 positive; MBP70-144-163 residues = 0.56-35.1 and 19, 8/14 positive; 19 kDa 1-16 residues = 0.60-8.76, 7/14 positive; 19 kDa 67-84 residues = 0.50-19.1, 8/14 positive; MBSE = 34.5-211.4; bovine IFNγ ELISA: only residue 122-138 = 1.13-6.03; residue 243-260 = 0.87-3.07; residue 307-328 = 0.60-10.36; PPD-B = 37.6-128.2; PPD-A = 2.4-24.2; MBSE = 34.5-211.4; bovine IFNγ ELISA: only residue 122-138 positive</td>
<td>103</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>Bovine IFNγ ELISA analysis of PPD-stimulated blood leukocytes of tuberculin negative cattle: of 98 IFNγ positive, 15 were culture positive</td>
<td>93</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PPD-B</td>
<td>Lyophilised, Biologics Unit, ADRI, Nepean, Ontario, Canada</td>
<td>Lymphocyte blastogenesis of elk: sensitivity = 70%; specificity = 74%</td>
<td>61</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>PPD-B</td>
<td>Lyophilised, Biologics Unit, ADRI, Nepean, Ontario, Canada</td>
<td>Lymphocyte blastogenesis of elk: sensitivity = 74%; specificity = 41%</td>
<td>61</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Old tuberculin</td>
<td>Biologics Unit, ADRI, Nepean, Ontario, Canada</td>
<td>Lymphocyte blastogenesis of elk: sensitivity = 45%; specificity = 75%;</td>
<td>61</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>Old tuberculin</td>
<td>Biologics Unit, ADRI, Nepean, Ontario, Canada</td>
<td>Lymphocyte blastogenesis of elk: sensitivity = 74%</td>
<td>61</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of PPD-stimulated whole blood (BCG vaccinated and <em>M. bovis</em> challenged animals): 19/20 IFNγ positive and culture positive</td>
<td>9</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>Lymphocyte blastogenesis: BCG vaccinated and <em>M. bovis</em> challenged animals: 16/20 IFNγ positive and culture positive</td>
<td>9</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>IL-2 bioassay: stimulation indices, culture positive = 7.5-18; culture negative = 2.0</td>
<td>9</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>38 kDa 1-27, 88-107, 122-138, 243-260, 307-328 residues; MBSE, PPD-B, PPD-A</td>
<td>Synthetic peptides, <em>M. bovis</em> sonic extract, PPD-B and PPD-A, CVL, Weybridge, UK</td>
<td>Lymphocyte blastogenesis stimulation indices (&gt;2.0 = positive); in PPD-B positive cattle 38 kDa residue 1-27 = 1.09-9.84; residue 88-107 = 0.93-15.49; residue 122-138 = 1.13-6.03; residue 243-260 = 0.87-3.07; residue 307-328 = 0.60-10.36; PPD-B = 37.6-128.2; PPD-A = 2.4-24.2; MBSE = 34.5-211.4; bovine IFNγ ELISA: only residue 122-138 positive</td>
<td>9</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNγ ELISA analysis of PPD-stimulated whole blood using manufacturer standard: sensitivity = 70% positive, compared to 90.4% of 84.4% for caudal fold test in 204 culture positive cattle</td>
<td>9</td>
</tr>
<tr>
<td>Organism</td>
<td>Antigen</td>
<td>Nature/antigen preparation</td>
<td>Reactivity</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td><em>M. bovis</em> (T/31/1376)</td>
<td>MBSE and PPD</td>
<td><em>M. bovis</em> sonic extract, PPD-B, CVL, Weybridge, UK</td>
<td>Lymphocyte blastogenesis stimulation indices (≥2.0) = positive; from early to late stages after experimental infection, Thy1 cells decreased, then increased. Ratios of CD4/CD8 increased, then decreased.</td>
<td>103</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>Standard, Commonwealth Serum Laboratories</td>
<td>MAb-based sandwich bovine IFNy ELISA analysis of PPD-stimulated whole blood using manufacturer standard.</td>
<td>49</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PPD-B</td>
<td>Standard, CVL, Lelystad, the Netherlands</td>
<td>Sandwich ELISA for bovine soluble IL-2 receptor: a analysis of PPD-stimulated PBMC. Only 1/51 positive in uninfected controls.</td>
<td>96</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PPD-B</td>
<td>Standard</td>
<td>Comparison of RT-PCR and MAb-based sandwich ELISA for bovine IFNy and IL-2 analysis of PPD-stimulated PBMC. Both are positive in culture positive cattle and correlate well with tuberculin skin test.</td>
<td>95</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> and <em>M. bovis</em></td>
<td>ST-CF, rESAT-6, PPD-B</td>
<td><em>M. tuberculosis</em> short-term culture filtrate, recombinant ESAT-6; <em>M. bovis</em> PPD-B, CVL, Weybridge, UK</td>
<td>Sandwich bovine IFNy ELISA analysis of ST-CF fractions, rESAT-6 and PPD-B PBMC from infected cattle: high response to &lt;10 kDa ST-CF antigens in early stages of infection; responses to both low and high molecular weight ST-CF antigens in later stages of infection; high responses to rESAT-6 in majority of naturally infected cattle equal to PPD-B</td>
<td>105</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>rMPB70, rMPB64, 81-100 and 174-190 residues of MPB70, 1-20, 41-60, 181-200 residues of MPB64</td>
<td>Recombinant MPB70 and MPB64, synthetic peptides of 81-100 and 174-190 residues of MPB70, and 1-20, 41-60, 181-200 residues of MPB64</td>
<td>Lymphocyte blastogenesis stimulation indices (≥2.0) = positive; and sandwich bovine IFNy ELISA analysis of rMPB70 and rMPB64, synthetic peptides of 81-100 and 174-190 residues of MPB70 and 1-20, 41-60, 181-200 residues of MPB64 and PPD-B PBMC from infected or immunised cattle: both high lymphoblastogenic and IFNy responses were detected in most cattle but no one antigen or epitope was recognised by all animals</td>
<td>71</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>rMPB69, 101-110 residues of MPB69, PPD-B</td>
<td>Recombinant MPB69 (fibronectin binding protein), synthetic peptides of 101-110 residues of MPB69, <em>M. bovis</em> PPD-B, CVL, Weybridge, UK</td>
<td>Lymphocyte blastogenesis stimulation indices (≥2.0) = positive; of <em>M. bovis</em>-specific bovine CD4 T cell clones and sandwich bovine IFNy ELISA analysis of rMPB69 and synthetic peptides of 101-110 residues of MPB69 and PPD-B-stimulation of whole blood from infected cattle; T cell clones and infected cattle responded positively to MPB69, but less than PPD-B, and 6/13 cattle responded to 101-110 residues of MPB69</td>
<td>72</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PPD-B</td>
<td><em>M. bovis</em> PPD-B, CVL, Weybridge, UK</td>
<td>Sandwich bovine IFNy ELISA and lymphocyte blastogenesis analysis of PPD-B-stimulated PBMC from infected cattle: generally increasingly positive IFNy and lymphocyte blastogenesis responses were detected continuously in 2/13 infected cattle at 7-42 days and at 14-42 days post infection, respectively.</td>
<td>12</td>
</tr>
<tr>
<td><em>M. bovis</em> WT</td>
<td>MBSE, rMPB70 PPD-B</td>
<td><em>M. bovis</em> sonic extract, live <em>M. bovis</em>, recombinant MPB70 and <em>M. bovis</em> PPD-B, CVL, Weybridge, UK</td>
<td>Sandwich bovine IFNy ELISA and lymphocyte blastogenesis analysis of CD8 and CD4 <em>M. bovis</em> T cell clones from PPD-B-stimulated PBMC from infected cattle: live <em>M. bovis</em>, MBSE and rMPB70 in descending order of stimulation significantly induced both IFNy and lymphoblastogenic responses of CD4 and especially endogenously processed antigens for CD8 T cells</td>
<td>70</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>CZ Veterinaria, Spain</td>
<td>MAB-based sandwich bovine IFNy ELISA analysis of PPD-B-stimulated whole blood: sensitivity = 84.5%, compared to single intradermal cervical test = 80.2%; sensitivity of combination of both tests = 92.9%, but with several false negative and numerous false positive results</td>
<td>50</td>
</tr>
<tr>
<td><em>M. bovis</em> AN5</td>
<td>PPD-B</td>
<td>CZ Veterinaria, Spain</td>
<td>MAB-based sandwich bovine IFNy ELISA analysis of PPD-B-stimulated whole blood from cattle of herds with 54.5% tuberculosis 'relativity': sensitivity = 100% with several false positives, compared to single intradermal cervical test = 89.3%</td>
<td>75</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>rMPB69, rMPB64, rMPB70, rESAT-6, PPD-B</td>
<td>Recombinant MPB59, MPB64, MPB70, ESAT-6, PPD-B, standard, Commonwealth Serum Laboratories</td>
<td>Sandwich bovine IFNy ELISA analysis of rMPB59, rMPB64, rMPB70, ESAT-6-stimulated whole blood from infected or BCG immunised cattle: only ESAT-6 and PPD-B stimulated high levels of IFNy; furthermore, only ESAT-6 stimulated a differential response between BCG-vaccinated and <em>M. bovis</em>-infected cattle</td>
<td>11</td>
</tr>
</tbody>
</table>
progressive development of nucleic acid-based molecular methods for this purpose. The ideal method of typing should be useful tools for advancing understanding of the dynamics of M. bovis transmission among animals. These techniques serve as the basis for improved tuberculosis control and eradication programmes at farm, regional, national and international levels.

The data in Table III provide compelling evidence for the immediate application of these methods to naturally occurring tuberculosis in any domestic or sylvatic mammal in almost any setting. The evolution of procedures shows the steady progress towards strain typing within sub-species of Mycobacterium. The ideal method of typing should be inexpensive, reproducible, rapid, easily performed and have direct application to clinical specimens. While none of the current methods fulfill all these criteria, the improvements achieved since the early 1990s have been remarkable. Most of these procedures are adaptable to modern laboratories and are useful tools for advancing understanding of the dynamics of M. bovis transmission among animals. These techniques serve as the basis for improved tuberculosis control and eradication programmes at farm, regional, national and international levels.

The broad spectrum of DNA fingerprinting methods for detecting and differentiating Mycobacterium spp. is fully characterised. Thus, this section is a review of the progressive development of nucleic acid-based molecular methods for this purpose. From the early DNA fingerprint analysis by restriction endonuclease procedures by Collins and de Lisle (19, 20), to the restriction fragment length polymorphism (RFLP) analysis by Thierry et al. (133), followed later by the spoligotyping methods of van Soolingen et al. (139) and Kamerbeck et al. (62), and now the multiplex PCR procedures of Sreevatsan et al. (129), the detection and differentiation of Mycobacterium spp. by nucleic techniques clearly hold great promise for epidemiological investigations at local, national and international population levels. Table III summarises the research in domestic and sylvatic mammals and provides a chronological and technological history of nucleic acid-based procedures not only to detect M. bovis, but to differentiate among members of the M. tuberculosis complex and identify strains infecting different populations of animals. This area of tuberculosis investigation has experienced a tremendous growth in productive research with direct application in laboratories on a world-wide basis.

### Table II (contd)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antigen</th>
<th>Nature/antigen preparation</th>
<th>Reactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovis</td>
<td>rMPB64, rMPB70, rMPB83, rESAT-6, 89-104 and 105-120 residues of MPB64, 1-16 residues of MPB70, 105-214 residues of MPB83, 1-16, 17-32, and 57-72 residues of ESAT-6, PPD-B</td>
<td>Recombinant MPB64, MPB70, ESAT-6, synthetic peptides 89-104 and 105-120 residues of MPB64, 1-16 residues of MPB70, 195-214 residues of MPB83, 1-16, 17-32, and 57-72 residues of ESAT-6, PPD-B, standard, Commonwealth Serum Laboratories</td>
<td>Sandwich bovine IFNγ ELISA and lymphocyte blastogenesis analysis of rMPB64, rMPB70, rMPB83, rESAT-6, 89-104 and 105-120 residues of MPB64, 1-16 residues of MPB70, 195-214 residues of MPB83, 1-16, 17-32, and 57-72 residues of ESAT-6 and PPD-B-stimulated PBMC from M. bovis-infected or BCG-vaccinated cattle: rMPB64, rMPB70, rMPB83 and rESAT-6 induced biogenesis and IFNγ secretion only in infected cattle. Importantly, a cocktail of promiscuous T cell epitopes 89-104 and 105-120 residues of MPB64, 195-214 residues of MPB83, 1-16, 17-32 and 57-72 residues of ESAT-6 detected responses only from M. bovis-infected cattle</td>
<td>141</td>
</tr>
<tr>
<td>M. bovis</td>
<td>PPD-B</td>
<td>M. bovis PPD-B, CVL, Weybridge, UK</td>
<td>Sandwich bovine IFNγ ELISA and lymphocyte blastogenesis analysis of PPD-B-stimulated whole blood from infected cattle: 8/9 calves were positive to IFNγ ELISA and 5/9 calves were positive to lymphocyte blastogenesis by 28 days post infection, while no antibodies were detected by ELISA</td>
<td>14</td>
</tr>
<tr>
<td>M. bovis</td>
<td>rESAT-6, rMPB64, rMPB70, rMPB83, rAg85A, B and C complex, PPD-B</td>
<td>Recombinant ESAT-6, MPB64, MPB70, MPB83, rAg85A, B and C complex, PPD-B, standard, Commonwealth Serum Laboratories</td>
<td>IL-2 bioassay, sandwich bovine IFNγ and TGFβ ELISA and lymphocyte blastogenesis analysis of rESAT-6, rMPB64, rMPB70, rMPB83, rAg85A, B and C complex, PPD-B-stimulated PBMC and draining lymph node cells from M. bovis-infected cattle: little difference was detected between PBMC and lymph node cells; ESAT-6 and PPD-B elicited the strongest responses of primarily CD4 and Tgd cells</td>
<td>108</td>
</tr>
</tbody>
</table>

**Abbreviations**
- ADRI: Animal Diseases Research Institute
- BCG: bacillus Calmette-Guérin
- CVL: Central Veterinary Laboratory
- ELISA: enzyme-linked immunosorbent assay
- hsp: heat shock protein
- iELISA: indirect ELISA
- IFNγ: interferon
- Ig: immunoglobulin
- IL: interleukin
- IFNγ: interferon
- IFNγ: interferon
- MAb: monoclonal antibody
- Mb: M. bovis
- Mb: M. tuberculosis
- PBMC: peripheral blood mononuclear cell
- PPD: purified protein derivative
- PPD-A: PPD derived from M. avium culture
- PPD-B: PPD derived from M. bovis culture
- RT-PCR: reverse transcriptase-polymerase chain reaction
- ST-CF: short-term culture filtrate
- TGFβ: transforming growth factor beta
- UK: United Kingdom
- Weybridge, UK

*References:*
incidence is likely to be greater. Much of the following information is based on the recent emergence of *M. tuberculosis* in Asian elephants in the USA (42).

**Epidemiology**

The recent emergence of *M. tuberculosis* in elephants in North America began with the death of two travelling circus elephants in 1996 (3). Between August 1996 and 3 June 2000, seventeen cases of *M. tuberculosis* were confirmed in Asian elephants in North America (42). Elephants from eight herds in Illinois, California, Arkansas, Missouri and Florida were affected. Contact had previously occurred between elephants in five of the herds. *Mycobacterium tuberculosis* was isolated from twelve elephants *ante mortem* and from five elephants *post mortem*. Five distinct strains of *M. tuberculosis* were identified, based on restriction fragment length polymorphism (RFLP) of isolates from eleven elephants. From this study, the prevalence of *M. tuberculosis* was estimated to be approximately 3.0% in the captive elephant population in North America (42).

**Clinical and pathological aspects**

Signs attributed to tuberculosis in elephants are mostly non-specific, e.g. inappetence, weight loss, reluctance to do strenuous work and occasionally subcutaneous ventral oedema. More typically, premonitory *ante-mortem* signs are absent. The major pathological changes in elephants with *M. tuberculosis* occur primarily in the lungs and thoracic lymph nodes with lesser involvement of extra-thoracic sites. In the less extensive cases, firm granulomatous nodules, sometimes with caseous foci, are noted in the bronchial lymph nodes and pulmonary tissue (Fig. 1). Elephants with extensive involvement of both lungs usually die with severe caseo-calcareous and cavitating lesions (Fig. 2). These often result in large pulmonary abscesses from which

![Fig. 1](Lung segments (after fixation in formalin) depicting an early case of tuberculosis with granulomas containing central caseation necrosis from a forty-five-year-old female Asian performing elephant from the United States of America (Bar = 1 cm). The larger nodules are surrounded by smaller satellite epithelioid granulomas. *Mycobacterium tuberculosis* was cultured from the lesions)

![Fig. 2](Advanced caseo-calcareous tuberculous pneumonia (Bar = 5 cm). Segment of pulmonary lobe from an Asian elephant (index case) from the 1996 outbreak of *Mycobacterium tuberculosis* in the United States of America. Photo: courtesy of Dr Hailu Kinde, California Animal Health and Food Safety Laboratory, University of California, Davis)

*M. tuberculosis* and opportunistic bacteria such as *Pseudomonas aeruginosa* have been isolated.

Characteristic histological findings include epithelioid granulomas with some giant cell formation in the initial lymph node and pulmonary lesions and extensive caseous and pyogranulomatous pneumonia in the advanced forms. Although sparsely distributed, acid-fast bacilli are more easily found in central areas of caseation in the lungs, rather than in the lymph nodes where they are typically rare. Bronchial and tracheal tuberculous plaques, and caseous and mucopurulent exudate in the nasal and trunk passages have been noted in both the early and late stages of tuberculosis, suggesting that the shedding of mycobacteria may occur at any stage of the disease.

**Diagnosis and control**

In 1997, a National Tuberculosis Working Group for Zoo and Wildlife Species in the USA formulated the *Guidelines for the Control of Tuberculosis in Elephants* (71) to specify criteria for the testing, surveillance and treatment of elephants for tuberculosis. Intradermal tuberculin tests to diagnose tuberculosis in elephants have correlated poorly with mycobacterial culture results, with high percentages of false-negatives in culture-positive animals. Currently, the test considered most reliable for diagnosis of tuberculosis in elephants is based on the culture of respiratory secretions obtained by trunk lavage (or "washes"). Sterile saline is instilled into the nostrils and then recovered in a plastic bag. Three samples are collected on separate days (26). Elephant herds in the USA are tested by this method on an annual basis or more frequently if cases of tuberculosis are detected or if the herd is known to have been previously exposed to tuberculosis. The flow sheet in Figure 3 presents the testing schedule in detail. A
<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Insertional element/restriction enzyme/gene</th>
<th>Reactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP</td>
<td>RFLP performed with DR, PGRS probed Alu I digests; 245 bp IS6110 probe of Pvu II digests</td>
<td>IS6110, DR and PGRS individually identified 17, 18 and 16 different RFLP types respectively from <em>M. bovis</em> isolates from cattle, feral deer, badgers, sheep, humans and swine, but the combination of the 3 techniques identified 39 distinct types adequate to indicate that the strains are not host restricted</td>
<td>128</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PFGE</td>
<td>PFGE of genomic DNA digested with <em>Dra I, Xba I, Spe I</em> or <em>Vsp I</em></td>
<td>PFGE identified 53 patterns from isolates of <em>M. bovis</em> from cattle, swine, deer, seals, badgers, possums and humans. <em>Dra I</em> was the most useful, producing fewer fragments of larger size which could be further sub-divided by <em>Spe I</em>. This was found to be highly discriminatory for epidemiological investigations</td>
<td>41</td>
</tr>
<tr>
<td><em>M. bovis</em>; <em>M. other species of Mycobacterium</em></td>
<td>PCR and multiplex PCR</td>
<td>Multiplex PCR for mtp40 and IS1081; multiplex PCR for 16S rRNA and MPB70; PCR for IS6110</td>
<td>mtp40 was not detected in <em>M. bovis</em>, while IS1081, 16S rRNA, MPB70 and IS6110 were detected in all <em>M. bovis</em> isolates, indicating that multiplex PCR of mtp40 and IS1081 may be useful for differentiating among members of the <em>M. tuberculosis</em> complex. IS6110 was reported in <em>M. gilvum</em> and <em>M. ulcerans</em> for the first time</td>
<td>69</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping</td>
<td>Spacere oligonucleotide typing – PCR amplification of spacers between the DRs</td>
<td><em>M. bovis</em> isolates from cattle and goats typed by spoligotyping revealed a common <em>M. bovis</em> caprine genotype which was useful in epidemiological studies of <em>M. bovis</em> transmission among humans and domestic livestock</td>
<td>55</td>
</tr>
<tr>
<td><em>M. bovis</em>, <em>M. other species of Mycobacterium</em></td>
<td>PCR, subtractive hydridisation</td>
<td>PCR of a 125 bp sequence apparently upstream of the isocitrate dehydrogenase gene of <em>M. bovis</em></td>
<td>PCR of a 125 bp sequence was detected only in the <em>M. tuberculosis</em> complex</td>
<td>60</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP</td>
<td>RFLP performed with RHS-245 pb and LHS-967 IS6110 probed Pvu II digests</td>
<td>The combination of RFLP performed with RHS-245 pb and LHS-967 IS6110 probed successfully differentiated multiple copy IS6110 isolates and was useful for differentiating among caprine and bovine <em>M. bovis</em></td>
<td>69</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PCR-SSCP</td>
<td>PCR-SSCP of a single base (C-to-G) point mutation of the Pzase (pncA) gene</td>
<td>PCR-SSCP of pncA differentiated 97 of 99 <em>M. bovis</em> isolates</td>
<td>123</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>BACTEC-spoligotyping</td>
<td>Spacere oligonucleotide typing – PCR amplification of spacers between the DRs was performed on BACTEC cultures at various growth indices</td>
<td>Within 10 days, 90.4% and 94.2% of BACTEC culture positive bovine samples at growth indices of =60 and =200 respectively yielded definitive spoligotyping patterns. Spoligotypes were obtained from 64.6% of culture-positive samples. Two spoligotypes accounted for 60.7% of culture positive samples</td>
<td>117</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping, RFLP</td>
<td>Spacere oligonucleotide typing – PCR amplification of spacers between the DRs and RFLP performed with DR and PGRS probed Alu I digests; 1356 pb IS6110 probe of Pvu II digests</td>
<td>IS6110, DR and PGRS identified 24 different RFLP types while spoligotyping revealed 15 different spoligotypes among <em>M. bovis</em> isolates from cattle, badgers and deer. Although RFLP typing was more discriminatory, spoligotyping was easier to perform, more rapid, easily documented and more suitable for large-scale screening purposes</td>
<td>62, 128</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping, RFLP</td>
<td>Spacere oligonucleotide typing – PCR amplification of spacers between the DRs and RFLP performed with DR and PGRS probed Alu I digests; 1356 pb IS6110 probe of Pvu II digests</td>
<td>PGRS closely followed by IS6110 RFLP was found to be the most sensitive method to identify polymorphisms among <em>M. bovis</em> isolates from cattle, sheep, goats, cats and wild boar</td>
<td>3</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP</td>
<td>RFLP performed with DR and PGRS probed Alu I digests; 1356 pb IS6110 probe of Pvu II digests</td>
<td>Combined DR and PGRS-RFLP types identified 93 different patterns which were successfully used for cluster analysis and epidemiological investigations</td>
<td>45</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping, RFLP</td>
<td>Spacere oligonucleotide typing – PCR amplification of spacers between the DRs and RFLP performed with DR and PGRS probed Alu I digests; RHI-245 pb IS6110 probe of Pvu II digests</td>
<td>The 273 <em>M. bovis</em> isolates from numerous domestic and sylvatic animals from 4 countries yielded: 23 IS6110 RFLP types, 35 DR types, 77 PGRS types and 35 spoligotypes, while the combination identified 89 different patterns which were useful for cluster and epidemiological investigation. IS6110 RFLP type analysis was adequate for strains having 3 copies, but IS6110 RFLP combined with PGRS was necessary for typing isolates with less than 3 copies. Spoligotyping was found to be rapid but lacking in sensitivity</td>
<td>33</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP, spoligotyping</td>
<td>IS6110-RHS RFLP of Pvu II digests, spoligotyping, and PGRS-RFLP of Alu I digests</td>
<td>For standardised recommendations for <em>M. bovis</em> isolates with 3 or more copies, IS6110-RHS RFLP was adequate. However, if the isolate has 2 or fewer copies, spoligotyping was recommended followed by PGRS-RFLP for maximum sensitivity in discriminating strains</td>
<td>32</td>
</tr>
</tbody>
</table>
documented in the comprehensive review of the use of molecular epidemiology for tuberculosis by Van Soolingen (138). Several methods of restriction enzyme analysis (REA), RFLP, random amplified polymorphic DNA (RAPD) analysis, PCR-ribotyping and multiplex PCR for several insertion sequences (IS) and genes have been developed and evaluated under experimental and field conditions, although most are laborious, technically demanding and not yet standardised. Fortunately, Cousins et al. have developed specific recommendations towards a standardised protocol for DNA

Table III (contd)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Insertional element/restriction enzyme/gene</th>
<th>Reactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em></td>
<td>PCR</td>
<td>PCR amplification of IS6110 from Cε-carboxypropylbetaine processed milk</td>
<td>Detection of <em>M. bovis</em> in bovine milk was enhanced to 94.1% as compared to 98.8% by standard glass bead processing</td>
<td>25</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>LCx</td>
<td>Amplification of 44 bp of <em>M. tuberculosis</em> proteic antigen 5 after 37 cycles followed by multiparticle enzyme immunoassay</td>
<td>LCx had a sensitivity of 83.9% and specificity of 100% compared to 85.7% sensitivity and 100% specificity of culture by BACTEC 460, resulting in a concordance of 81.1% of LCx versus BACTEC 460</td>
<td>16</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP, spoligotyping</td>
<td>RFLP performed with DR and PGRS probed Alu I digests; spoligotyping</td>
<td>Of 154 <em>M. bovis</em> isolates, mainly from South America, spoligotyping identified 41 different types, DR and PGRS identified 42 types each, while combining spoligotyping with DR and PGRS identified 88 types. Spoligotyping was recommended as the initial step, followed by DR and PGRS RFLP analysis for cluster analysis</td>
<td>151</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RFLP, (GTG)PCR, ERIC primers for PCR, PCR ribotyping</td>
<td>IS6110 RFLP with 0.45 kb Bam HI-Sai I fragment performed on Pvu II digests; primers for (GTG)5, ERIC and 3' end of 16S and 5' 23S of mt ribotyping followed by Hae III restriction were used for PCR amplification</td>
<td>IS6110 RFLP identified 8 patterns, ERIC detected 7 patterns, and (GTG), discerned 8 patterns, while PCR interspacer ribotyping was not useful</td>
<td>124</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>500 bp PCR</td>
<td>PCR amplification with JB21-JB22 primers to produce a 500 bp probe for hybridising Pvu II digests</td>
<td>100% of 121 <em>M. bovis</em> isolates were detected</td>
<td>115</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping, RFLP</td>
<td>Spoligotyping; IS6110 and IS1081 used to probe Pvu II digests</td>
<td>Spoligotyping identified 9 patterns, IS6110 differentiated a single and a 5 copy pattern, while IS1081 did not detect any differential patterns in <em>M. bovis</em> isolates from 63 wild boar and 16 cattle. Spoligotyping was the most useful procedure for cluster analysis and epidemiological investigations</td>
<td>126</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Spoligotyping, RFLP</td>
<td>Spoligotyping; IS6110 and IS1081 used to probe Pvu II digests</td>
<td>IS6110 RFLP identified 6 patterns, ERIC detected 7 patterns, and 124 IS6110 RFLP identified 8 patterns, ERIC detected 7 patterns, and 124 patterns in <em>M. bovis</em> isolates from 63 wild boar and 16 cattle. Spoligotyping was the most useful procedure for cluster analysis and epidemiological investigations</td>
<td>102</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>Species-Specific gyrB-PCR-RFLP</td>
<td><em>Chemistry</em> and Taq I restriction of the PCR product of the primer set 756-A and 1410-A</td>
<td>Species-specific gyrB-PCR-RFLP differentiated all members of the <em>M. tuberculosis</em> complex</td>
<td>63</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>RAPD-PCR</td>
<td>Four random primers and two selected primers were used for RAPD-PCR</td>
<td>Although RAPD-PCR identified 98 different patterns of 99 genetically dispersed isolates from Mexico, the method was not able to differentiate <em>M. bovis</em> isolates by epidemiological details or identify a common source of infection</td>
<td>82</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Species-specific multiplex PCR</td>
<td>Multiplex PCR of repeat-sequence of <em>M. tuberculosis</em> 500 bp followed by solid-phase probe capture hybridisation</td>
<td>Multiplex PCR of <em>M. bovis</em> 100 bp detected down to 4 CFU equivalents in milk and nasal swabs of naturally and experimentally infected cattle</td>
<td>129</td>
</tr>
</tbody>
</table>
fingerprinting of *Mycobacterium* spp. that, in conjunction with clinical field investigations, are expected to improve research addressing the maintenance and transmission of *M. bovis* in domestic and sylvatic animal populations (32). For *M. bovis* isolates with three or more copies, IS6110-RHS RFLP is adequate, even though IS6110-LHS RFLP has been shown to be more sensitive. However, if the isolate has two or fewer copies of IS6110, spoligotyping is recommended followed by polymorphic GC-rich repeat sequence (PGRS)-RFLP analysis for maximum sensitivity in discriminating strains (32), using computer software for standardised computer-assisted analysis and documentation of the banding patterns. Use of standardised protocols will facilitate global comparisons and tracking of the transmission dynamics of *M. bovis*. Combinations of multiple RFLP analysis and spoligotyping were found to yield the best differentiation among field strains of *M. bovis*. Further refinements for molecular fingerprinting of *Mycobacterium* spp. are underway and include genome-based fluorescent amplified-fragment length polymorphism (FAFLP) using two restriction endonucleases (51) and analysis of whole genomic relatedness through high-density DNA microarrays (7). Both these procedures offer an enhanced ability to differentiate among strains without having to use technically demanding and laborious procedures.

Conclusions

Modern technology and molecular biology have affected virtually every form of discovery, confirmatory or exclusionary diagnostic test, both in vivo and in vitro, often significantly enhancing the quality of the assays or providing a better understanding of host and/or *M. bovis* interactions. Valid study designs to evaluate diagnostic tests in the laboratory as well as under field conditions are essential for accepting or rejecting completely new tuberculosis diagnostics. Despite protein purification and molecular cloning, no individual mycobacterial proteins or cocktail of recombinant proteins have been found to eliminate the specificity problems of PPD or to elicit DTH-like responses. However, a *M. tuberculosis* 9 kDa protein, DPPD, was recently found to detect a strong and specific DTH having no cross-reactivity with other pathogenic or environmental mycobacteria, thus providing the first putative specific and sensitive alternative reagent for the diagnosis of tuberculosis. Thus, modern molecular approaches to identify and clone DTH diagnostic antigens may provide new reagents for detecting *M. bovis*-infected livestock with equal or greater sensitivity and specificity than current PPD antigens. This may even allow vaccination of animals against tuberculosis while discriminating *M. bovis* infected from vaccinated populations.

Assays based on PCR using formalin-fixed paraffin-embedded sections with typical tuberculous lesions are highly specific for *M. tuberculosis* complex mycobacteria and more rapid than bacterial culture by three to four weeks. The use of immunohistochemical and PCR-based procedures offers rapid, specific confirmatory tests for *M. bovis* infection, especially on sections or tissues with typical tuberculous lesions and acid-fast bacilli, or when herd history, tuberculin test results and necropsy findings are available.

Most laboratories recommend the use of both an agar-based medium (Middlebrook 7H11) and an egg-based medium (Stonebrink or Löwenstein-Jensen with pyruvate) for primary isolation. Modified Middlebrook 7H11 medium, B83 medium and radiometric detection have all improved the recovery rate and speed of culture when used in conjunction with standard specimen processing procedures. Identification of isolates may be performed by standard procedures including acid-fast staining, colony morphology and a panel of biochemical tests such as susceptibility to 2-thiophene carboxylic acid hydrazine (TCH) and isoniazid which require an additional three to four weeks. However, the use of monoclonal antibodies or genetic techniques now allow definitive identification of *M. bovis* and DNA fingerprinting of the isolate to be performed in one day.

Extensive evaluation of purified specific antigens as well as crude *M. bovis* antigens for the serological detection of cattle infected with *M. bovis* has revealed that the great majority of serological assays are not as accurate as in vivo or in vitro cell-mediated immunity tests. Serological tests for tuberculosis should be used to complement diagnostics based on cell-mediated immunity.

The comparative sandwich bovine IFNγ ELISA for *M. bovis* (using PPD-B and PPD-A) has probably had the most impact on improving the specificity and sensitivity of simplified and rapid in vitro cell-mediated immunity diagnostic tests (143). This test has been evaluated world-wide and found to be useful as a primary tuberculosis test under some conditions, and as a complement to skin-testing in most countries. Adaptations and refinements of the sandwich bovine IFNγ ELISA concept have steadily allowed investigators to explore the spectrum of bovine host cell-mediated immune responses of T cell subsets to a wide variety of crude and highly purified, recombinant or synthetic antigens with specific epitopes. The T cells appear to recognise a varying array of antigens, perhaps low molecular weight secreted antigens, during the stages of *M. bovis* infection of cattle, especially during early stages of infection. In the future, these early-stage secreted antigens could potentially be applied for diagnosis of active bovine tuberculosis.

Methods of DNA fingerprinting have evolved rapidly and deepened the understanding of naturally occurring tuberculosis in domestic or sylvatic mammals. Several methods of REA, RFLP, RAPD, PCR-ribotyping and multiplex PCR for several insertion elements and genes have been developed and evaluated under experimental and field conditions. Fortunately, specific recommendations towards a
standardised protocol for DNA fingerprinting of *Mycobacterium* spp. have been made that are expected to improve understanding of the epidemiology, maintenance and transmission of *M. bovis* in conjunction with clinical field investigations in domestic and sylvatic animal populations.

Significant progress has been made to improve the quality of the in vivo and in vitro tuberculosis diagnostics for domestic and sylvatic animals and additional progress is expected in the near future.

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**Diagnostic *in vivo* et *in vitro* de l’infection due à *Mycobacterium bovis***

L.G. Adams

Résumé

Le test intradermique d’hypersensibilité retardée utilisant un dérivé protéique purifié issu de la mise en culture de *M. bovis* ou de *M. avium* est l’épreuve la plus utilisée pour le dépistage de la tuberculose ou le diagnostic de l’infection à *M. bovis* chez les bovins. De nombreuses améliorations ont été apportées au test tuberculinique initial et les méthodes moléculaires visant à identifier et à cloner les antigènes pourront accrêoir la spécificité et la sensibilité des tests intradermiques d’hypersensibilité retardée. Récemment, grâce aux derniers progrès technologiques, de nouvelles techniques diagnostiques *in vitro* fondées sur la détection d’anticorps, sur l’immunité à médiation cellulaire ou sur des sondes nucléiques ont été mises au point, assurant un diagnostic plus rapide que la culture bactériologique. Le choix de la méthode diagnostique doit être effectué en fonction de la population cible (animaux apparemment sains ou troupeau présentant une forte prévalence de cas cliniques) et de l’objet de la recherche (sélection d’animaux sains ou confirmation de l’infection dans les cas suspects). D’autre part, les épreuves diagnostiques doivent être évaluées en faisant appel à des groupes témoins soigneusement sélectionnés, dont la répartition entre animaux infectés et non infectés soit représentative de la population cible.

Mots-clés


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**Diagnóstico *in vivo* e *in vitro* de la infección debida a *Mycobacterium bovis***

L.G. Adams

Resumen

La prueba intradérmica de hipersensibilidad retardada con uso de un derivado proteico purificado a partir de un cultivo de *M. bovis* o *M. avium* es la técnica más utilizada para diagnosticar la tuberculosis o detectar la infección por *M. bovis* en el ganado vacuno. La prueba original de la tuberculina ha ido experimentando
sucesivas mejoras, y el uso de técnicas moleculares para identificar y clonar antígenos puede dotar a esta prueba dermica de mayor especificidad y sensibilidad. Los últimos avances tecnológicos han propiciado la elaboración de nuevas técnicas in vitro, como las de diagnóstico por anticuerpos, por la inmunidad mediada por células o por reconocimiento de ácidos nucleicos, métodos todos ellos que permiten diagnosticar la enfermedad con más prontitud que un cultivo bacteriológico. Para elegir una técnica de diagnóstico conviene tener muy presente tanto la población que se analiza (animales en apariencia sanos o rebaños con elevada prevalencia de casos clínicos, por ejemplo) como el objetivo de los análisis (por ejemplo, la selección de animales sanos o la confirmación de la infección en casos sospechosos). Por otra parte, a la hora de evaluar una prueba de diagnóstico es necesario elegir con suma cuidado una población de control que sea representativa de la población problema (en cuanto a la proporción relativa de ejemplares infectados y no infectados).

Palabras clave

References


