Comparison of various diagnostic methods for the detection of *Mycoplasma bovis*

K. SACHSE, H. PFÜTZNER, H. HOTZEL, B. DEMUTH, M. HELLER and E. BERTHOLD *

**Summary:** Mycoplasma bovis, the main causative agent of mycoplasmal mastitis, arthritis and pneumonia in cattle, causes considerable economic losses. Veterinary hygiene measures would be most effective if introduced at an early stage, especially the culling of cows shedding the pathogen for the control of mastitis. It is therefore crucial to ensure that diagnostic methods are available which can perform rapid and specific detection of the agent at acceptable costs.

Six different detection methods have been compared and evaluated in terms of performance parameters and suitability for routine diagnosis. Conventional *M. bovis* isolation and identification from culture is the only technique used for routine diagnosis at present. However, this process is rather laborious and time-consuming, and final results are available only after several days. Enzyme-linked immunosorbent assay (ELISA) techniques can be used to screen for *M. bovis* antibodies or antigens in clinically-diseased animals. Detection of the agent in subclinical cases was accomplished in pre-incubated samples by an antigen capture ELISA involving a monoclonal antibody. Whole-cell protein patterns generated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis were used to identify and classify field isolates. Nucleic acid hybridisations using probes of defined specificity were conducted both as filter dot blot assay and in solution with ribosomal ribonucleic acid as the target. The latter was found to be potentially suitable for the screening of biological samples, although problems due to high background and reduced specificity remained. Finally, the presence of *M. bovis* cells in culture supernatant and in milk samples was demonstrated using the polymerase chain reaction. This procedure is potentially superior to all others currently available, due to its high sensitivity, specificity and speed. However, a number of practical problems must be solved prior to full-scale introduction of this technique for routine diagnosis.


**INTRODUCTION**

*Mycoplasma bovis* has been described as the aetiological agent of severe bovine diseases, such as mastitis of cows, arthritis and pneumonia of calves and young cattle, as well as genital disorders in bulls and cows (18, 21, 26, 27). Mycoplasmal diseases are
characterised by severe clinical symptoms and resistance to therapy. No effective immunoprophylactic measures are known. These circumstances make effective control of the infection very difficult, and the economic losses caused are often considerable (1, 4, 12). It should be further noted that, in addition to specific control measures, the observation of a high standard of veterinary hygiene is an essential prerequisite for the prevention of *M. bovis* infections. Due to non-specific clinical symptoms and great variation in epizootiology and pathogenesis, each disease caused by *M. bovis* requires a specific diagnostic approach and specific control measures. This situation is illustrated in Table I, which also contains a summary of the diagnostic methods used.

### Table I

**Diagnostic approaches to Mycoplasma bovis infections**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Control</th>
<th>Diagnosis</th>
<th>Diagnostic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastitis</td>
<td>Culling of shedders</td>
<td>Detection of cows shedding the pathogen via milk (single-animal diagnosis)</td>
<td>Isolation and culture; Antigen capture ELISA; PCR*</td>
</tr>
<tr>
<td>Arthritis, pneumonia</td>
<td>Herd entry restrictions (no culling of shedders)</td>
<td>Screening and monitoring (herd diagnosis)</td>
<td>Antibody ELISA (isolation and culture)</td>
</tr>
<tr>
<td>Genital disease</td>
<td>Decontamination of semen</td>
<td>Detection of animals shedding the pathogen via genital tract (single-animal diagnosis)</td>
<td>Isolation and culture; Antigen capture ELISA*; PCR*</td>
</tr>
</tbody>
</table>

* no use in routine diagnosis at present

ELISA: enzyme-linked immunosorbent assay

PCR: polymerase chain reaction

At present, mastitis caused by *M. bovis* is controlled mainly by the culling of all cows shedding the agent via milk. Detection of shedders at a very early stage of disease is crucial for this method of control, and single-animal diagnosis is essential if control is to be effective. In the case of arthritis and pneumonia caused by *M. bovis*, control can be achieved through early herd entry restrictions based on the findings of comprehensive livestock monitoring (i.e. herd diagnosis), and shedders no longer need to be selectively culled. The main control measures for genital diseases caused by *M. bovis* include mycoplasma decontamination of semen (17, 28, 29) and, if inevitable, culling of shedders. As for mastitis, early identification (i.e. single-animal diagnosis) of all animals shedding *M. bovis* via the genital tract is a necessary precondition for successful control. Consequently, there is a need to develop new diagnostic procedures which are not only more rapid but also more specific than existing techniques.

In the present paper, the authors describe three diagnostic methods applicable for the detection of *M. bovis* and other mycoplasmas, and compare the main parameters of performance on the basis of experience gained through several research projects in recent years. The main results and conclusions are summarised in Table II.
### TABLE II

**Comparison of diagnostic methods for Mycoplasma bovis**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conventional Culture</th>
<th>ELISA detection of antibodies</th>
<th>Methods</th>
<th>Protein antigen-based</th>
<th>Hybridisation DNA, RNA probe</th>
<th>Polymerase chain reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of information provided</td>
<td>presence of viable germs</td>
<td>presence of M. bovis antibodies</td>
<td></td>
<td>presence of 26 kDa surface antigen</td>
<td>presence of specific DNA or RNA sequences</td>
<td>presence of specific DNA sequences</td>
</tr>
<tr>
<td>Specificity</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Sensitivity (CFU/ml)</td>
<td>$10^3$-$10^4$</td>
<td>100-fold of IHA</td>
<td>$10^3$-$10^5$</td>
<td>$10^6$</td>
<td>$10^5$-$10^6$</td>
<td>as low as $10^0$</td>
</tr>
<tr>
<td>Time required</td>
<td>5-10 days</td>
<td>2 days</td>
<td>1-3 days</td>
<td>cultivation plus 4-5 h</td>
<td>2 days</td>
<td>1 day</td>
</tr>
<tr>
<td>Ease of operation</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Advantage</td>
<td>simultaneous rapid processing detection of other mycoplasmas</td>
<td>rapid processing of many samples</td>
<td>rapid processing of many samples</td>
<td>strains can be identified and classified</td>
<td>high and defined specificity</td>
<td>rapidity and specificity</td>
</tr>
<tr>
<td>Drawback</td>
<td>possible impairment by bacterial contamination</td>
<td>no basis for control of disease</td>
<td>pre-incubation required for good sensitivity</td>
<td>standardisation difficult</td>
<td>background problems with biological material</td>
<td>danger of outside contamination</td>
</tr>
<tr>
<td>Direct screening of biological samples</td>
<td>yes</td>
<td>serum only</td>
<td>yes</td>
<td>no</td>
<td>possible</td>
<td>yes</td>
</tr>
<tr>
<td>Suitability for routine laboratory use</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

++ very good
+ good
± adequate
− poor

ELISA: enzyme-linked immunosorbent assay
MAb: monoclonal antibody
DNA: deoxyribonucleic acid
RNA: ribonucleic acid
IHA: indirect haemagglutination assay

### CONVENTIONAL METHODS OF DETECTION

To date, routine diagnosis of *M. bovis* infection has been performed exclusively by conventional culture methods subsequent to mycoplasma isolation (11, 30). However, these methods are laborious and time-consuming and can be impaired by bacterial contamination of the samples. The most serious drawback of such methods is that the final results are available only after several days – as long as five to ten days in unfavourable circumstances. Nevertheless, culture methods will remain important for
routine use in laboratories, as the high specificity and sensitivity ($10^{1}-10^{2}$ colony-forming units [CFU]/ml) of these tests ensure reliable detection of the pathogen from a wide variety of clinical specimens. *M. bovis* isolation can be a useful tool for single-animal diagnosis and will serve as the basis for control of mycoplasmal mastitis until more rapid routine diagnostic assays are introduced. Furthermore, culture methods are suitable for simultaneous detection of several mycoplasma species present in the same sample.

As a conventional alternative to antigen detection, antibodies against *M. bovis* in blood and milk can be detected by enzyme-linked immunosorbent assay (ELISA) techniques (5, 40). These are generally less labour-intensive and time-consuming than culture methods and allow the screening of large numbers of samples; results are normally available within two days. The authors developed an ELISA for *M. bovis* antibodies using whole-cell antigen of the agent for solid-phase coating (7). The assay has proved sufficiently specific and the sensitivity of detection ($10^{5}-10^{6}$ CFU/ml) was 100 times greater than with other serological methods, such as film inhibition and indirect haemagglutination. However, the application of antibody detection ELISAs is limited by the fact that antibody titres only emerge ten to fourteen days after the onset of disease. Consequently, the pathogen cannot be detected during the incubation period. The attainable sensitivity is insufficient for reliable identification of all animals shedding *M. bovis*. Nevertheless, ELISA detection of antibodies can be a useful method for regular screening of *M. bovis*-free herds for trade purposes and livestock monitoring in general.

**PROTEIN ANTIGEN-BASED METHODS OF DETECTION**

The specificity of conventional antigen ELISAs can be improved by targeting a particular protein rather than the whole complex of cellular proteins (6, 14). Earlier investigations suggested involvement of a 26 kDa surface protein, p26, in the cell adherence process of *M. bovis* (35). Using a monoclonal antibody directed against the p26 antigen, the authors developed an antigen capture ELISA which was successfully employed for the detection of *M. bovis* in bovine milk (3, 13). The assay has proved highly specific, with cross-reactions being confined to *M. agalactiae*. The latter should not be relevant in the diagnosis of bovine diseases, as *M. agalactiae* is known to be strongly adapted to sheep and goats. Bacterial contamination of the sample was not found to impair *M. bovis* detection in any way. Since the whole ELISA procedure is sufficiently rapid (one to three days) and can be conducted in normally equipped diagnostic laboratories, it is well suited for screening examinations involving large numbers of samples. Given the comparatively low sensitivity ($10^{5}$ CFU/ml), the normal protocol can only be recommended for the screening of milk samples from cows with clinical mastitis. However, pre-incubation of the samples for 24 h or 48 h can improve detection limits by two orders of magnitude. In this variant, the antigen capture ELISA has proved suitable for single-animal diagnosis of *M. bovis* mastitis by the screening of diseased cows and high-level shedders, as well as for complete herd monitoring.

Whole-cell protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and/or immunoblotting is widely used to characterise the antigen repertoire of individual bacterial strains, including mycoplasmal isolates (8, 20, 25, 39). The procedure is characterised by high specificity, which allows the
differentiation of distinct groups within a species. On the basis of individual banding patterns, it is possible to identify and classify, or trace the presumed origin of strains. Such analyses can provide useful indications concerning antigenic relationships within and between species. For an assessment of the scope of intraspecies antigen variability the authors compared whole-cell protein SDS-PAGE and immunoblot patterns for thirty-four field isolates of *M. bovis* (34). The main conclusion was that antigenic variation within this group of strains was mostly confined to quantitative differences; the concentrations of certain antigen clusters in the molecular weight ranges of 64-68 kDa, 55 kDa and 26 kDa varied between individual strains. It can be assumed that this phenomenon is part of a very versatile and complex mechanism of surface antigen variation in *M. bovis* as recently discovered by Rosengarten and colleagues (33). As far as the use of whole-cell protein analysis for diagnostic purposes is concerned, several essential limitations must be taken into account, including:

- low sensitivity (approximately $10^6$ CFU/ml)
- the impossibility of direct *M. bovis* detection in clinical material
- the necessity for cultivation of the isolate strain in each case
- the difficulty of standardisation.

On the other hand, the information provided by a protein pattern is quite extensive with regard to all major antigens, thus making whole-cell protein analysis a suitable tool for research in the fields of epizootiology and pathogenesis.

**DNA-BASED DIAGNOSIS OF MYCOPLASMA INFECTION**

In recent years, there has been a virtual revolution in methods for the detection of bacteria, viruses and other micro-organisms, as a consequence of the use of new molecular biology techniques. In view of the potential of these methods, it is widely agreed that deoxyribonucleic acid (DNA)-based diagnosis of mycoplasmal diseases is emerging as the diagnostic tool of choice for the future. A large number of different methodological approaches to mycoplasma detection are described in the literature, involving hybridisation assays with DNA or ribonucleic acid (RNA) probes (9, 10, 16, 19, 22, 24, 31, 32, 36, 37, 38) or DNA *in vitro* amplification by the polymerase chain reaction (PCR) (2, 23).

The authors have developed a variety of specific nucleic acid probes for the detection of *M. bovis*, which have been used in filter dot blot and solution hybridisation. Furthermore, primer sets derived from these probes have been employed for PCR detection of the agent. This research is described in detail elsewhere in this issue of the Review (15). The results indicated that although filter dot blot hybridisation is highly specific for extracted DNA of *M. bovis*, it is not suitable for assaying biological samples, as the selective binding of DNA to the membrane is severely disturbed by proteins and other substances present in the material. Solution hybridisation with RNA probes was used by the authors to screen large numbers of experimentally-infected milk samples. Although the results achieved to date have not been completely satisfactory (Table II), this method is still considered potentially applicable for the routine diagnosis of samples containing mycoplasmas; particular advantages of such a procedure would be rapidity (one or two days) and high specificity. However, sensitivity of detection was found to
range between $10^5$ and $10^6$ CFU/ml in our experiments, which is not usually sufficient to detect the low levels of mycoplasma which occur during the incubation period or in chronically-diseased animals. Moreover, radioactive labelling of the probes is still required in most cases to attain reasonable sensitivity; such a practice is detrimental to any normal use in the laboratory. The sensitivity of this method cannot be expected to be increased much further because of the background problems with biological samples referred to above.

As with hybridisation assays, PCR detection of *M. bovis* in milk was seriously hampered by the interference of proteins, fats and probably also ions present in the milk. Various approaches were attempted to minimise this interference, and in a series of experiments involving protease pre-treatment it was shown that direct detection of *M. bovis* from milk is possible using PCR. This procedure detected $10^7$ CFU/ml, but further optimisation is possible. While there can be no doubt that PCR is potentially the most sensitive of the methods discussed in this paper, it must be emphasised that there are often unrealistic expectations concerning the detection limit of this technique. For PCR, only a few microlitres of the sample can be taken, so that even if at optimal conditions the DNA of a single mycoplasma cell is amplified and detected, the overall detection limit will be approximately $10^3$ CFU/ml. The latter can be lowered only if DNA pre-enrichment steps are introduced. Nevertheless, PCR should be the method of choice for mycoplasma detection from biological samples, as it can be performed very rapidly (in one day) and is extremely specific for the target species; even differentiation of individual strains within a species is possible if appropriate probes are available. The comprehensive use of PCR could open up new possibilities for the control of diseases caused by *M. bovis* and other mycoplasmas.

ACKNOWLEDGEMENT

The authors wish to thank the *Deutsche Forschungsgemeinschaft*, which funded part of this study (Grant No. Ki 215/5-1).

* * *
On a comparé dans ce but six méthodes différentes de détection et évalué leurs performances ainsi que leur valeur pour un diagnostic de routine. La technique conventionnelle d'isolement et d'identification en culture cellulaire est la seule utilisée à ce jour pour les diagnostics de routine ; toutefois, le procédé est fastidieux et long et il faut attendre plusieurs jours avant d'avoir les résultats. La technique enzyme-linked immunosorbent assay (ELISA) peut servir à repérer des anticorps ou antigènes M. bovis chez des animaux présentant des manifestations cliniques. La détection du contaminant en phase subclinique a été réalisée dans des prélèvements ayant subi une pré-incubation, à l'aide d'un test d'immunocapture ELISA utilisant un anticorps monoclonal. Les profils observés après électrophorèse sur gel au dodécylsulfate de sodium-polyacrylamide de protéines brutes, ont permis l'identification et le typage d'isolats de terrain. Des hybridations de l'acide nucléique au moyen de sondes de spécificité bien définie ont été réalisées à la fois en test «dot blot» sur papier filtre et en solution utilisant l'acide ribonucléique ribosomique comme cible. Cette dernière méthode s'est avérée convenir au dépistage des mycoplasmes dans les prélèvements biologiques, bien que son bruit de fond important et sa spécificité réduite posent encore des problèmes. Enfin, la présence de M. bovis dans le surnageant des cultures et dans des prélèvements de lait a été révélée à l'aide de la technique d'amplification en chaîne par polymérase. Ce procédé semble se distinguer de toutes les méthodes actuellement disponibles par une sensibilité, une spécificité et une rapidité de résultat élevées. Cependant, de nombreux problèmes pratiques restent à résoudre avant toute application aux diagnostics de routine à grande échelle.

proteínas brutas, tales como las generadas por electroforesis en gel de dodecисulfato de sodio poliacrilamida, permitieron la identificación y caracterización molecular de aislados de terreno. Se realizaron también hibridaciones del ácido nucleico por medio de sondas de especificidad bien definida, a la vez como prueba de filtraje «dot blot» y en solución con el ácido ribonucleico ribosomal como blanco. Este último método parece ser el conveniente para las investigaciones en las muestras biológicas, aunque subsisten problemas importantes debidos a una fuerte interferencia y una especificidad reducida. Por último, se estableció la presencia de células M. bovis en cultivos sobrenadantes y muestras de leche por medio de la técnica de amplificación en cadena por polimerasa. Este procedimiento parece distinguirse de todos los demás disponibles en la actualidad, por sus elevadas sensibilidad, especificidad y rapidez. Sin embargo, quedan por resolver muchos problemas prácticos antes de cualquier aplicación a gran escala en diagnósticos de rutina.

PALABRAS CLAVE: Amplificación en cadena por polimerasa - Diagnóstico de laboratorio - ELISA - Especificidad - Hibridación del ácido desoxirribonucleico - Mycoplasma bovis - Pleuroneumonía contagiosa bovina - Sensibilidad - Sondas nucleicas.

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