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Serodiagnosis of contagious bovine pleuropneumonia by immunoblotting

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Contagious bovine pleuropneumonia (CBPP) is a bacterial disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (MmmSC). CBPP is listed by the World Organisation for Animal Health (OIE) as notifiable and notification is also mandatory on a national level. This results in strict health control measures, including depopulation and restrictions on livestock movements and exports.

Once a worldwide livestock affliction, CBPP now only persists in Africa and some areas of Asia. Europe has been considered CBPP-free since 1900 in spite of a few local outbreaks. The last outbreak was widespread, affecting all of south-western Europe between 1980 and 1999. It resulted from the insidious spread of a new, less virulent variant of MmmSC (Nicholas *et al.*, 1996).

The attenuated and chronic forms of PPCB are difficult to detect. They are the main source of spread and can often only be detected by systematic serological testing. These subclinical forms of the disease are typical of infections with the contemporary European variant of PPCB. The complement fixation (CF) test and competitive ELISA are the OIE-approved serological tests. However, their lack of sensitivity makes them inefficient in cases of low prevalence and in infection with the less immunogenic European strain of CBPP. Only the routine use of the immunoblotting test (IBT) in Europe made it possible to eliminate the disease in the last affected areas in Portugal in 1999 when all other strategies had failed (Nicholas *et al.*, 2008). This highly sensitive and highly specific test (Schubert *et al.*, 2011) is recommended by the OIE as a confirmatory test. However, as it is currently described in the OIE manual (2008), IBT lacks reproducibility and robustness. The only international IBT inter-laboratory proficiency test (ILPT), carried out in 2009, showed that results vary widely among laboratories. Here, we propose some improvements to standardise reagent production as well as the serodiagnosis procedure to increase reproducibility and to set up quality controls.

Reagent production: selecting the strain, preparing the antigen and controlling quality

The choice of the strain to use is an extremely critical point. The use of the B103 strain, the reference strain used by Regalla *et al.* in 1999 to develop the IBT method, is highly recommended and should be used to ensure consistent results among laboratories. The antigen is then prepared from a culture grown in mycoplasma broth medium (Poumarat *et al.*, 1991) and concentrated to 10^{11} to 10^{12} CFU/mL (Table 1). It is absolutely essential to ensure that the cultured strain correctly expresses the five specific proteins targeted in the IBT, which are 110, 98, 95, 60-62 and 48 kDa in size, as defined by Gonçalves *et al.* (1998). The expression levels of these proteins can vary according to the MmmSC strain used and the selected clone. Thus, prior to use, each antigen batch must be assessed with positive and negative reference sera (Table 1).

The culture must be checked for contamination by other mycoplasmas. Trace levels of contamination can be detected using the colony blot method (Gaurivaud *et al.*, 2004) with a MmmSC-specific monoclonal antibody (Brocchi *et al.*, 1993). The mycoplasma protein concentrate can be aliquoted and stored at -20°C up to one year.

Preparation of antigen strips

The OIE manual recommends separating the extracted proteins on a 5-15% gradient-resolving polyacrylamide gel. This polyacrylamide gradient ensures optimal separation of all the MmmSC proteins and was used by Gonçalves *et al.* (1998) to identify the set of five consensus antigenic proteins specific to



MmmSC. However, protein separation is optimal only if proteins migrate to the gel concentration appropriate for their molecular mass. For this reason, in practical terms, reproducibility is often difficult to control, mainly for two proteins, the 95 and 98 kDa proteins. Use of a 7% acrylamide gel (Schubert *et al.*, 2011) offers a compromise, with good separation of the proteins and better reproducibility between batches of antigen strips. To reduce the preparation time, commercial, ready-to-use gels can be used, such as Invitrogen NUPAGE 7% Tris-Acetate gels



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and Bio-Rad Mini-Protean TGX Any kD gels (Figure 1A). Mini-Protean TGX Any kD gels were chosen for their homogeneity in band intensity for the 95 and 98 kDa proteins in IBTs with positive sera. It should be noted that commercial gels do not necessarily guarantee the separation of proteins in the 95 - 98 kDa size range (e.g. 7.5% Mini-Protean TGX gels from Bio-Rad). After electrophoresis, the proteins are transferred to a

nitrocellulose membrane using a semi-dry transfer protocol (Gravel, 2002), which has several advantages over the wet transfer protocol: it is less time-consuming, several gels can be transferred simultaneously and a standard power supply can be used because a lower voltage is required (Kurien and Scofield, 2006; MacPhee, 2010). The transfer of proteins ranging from 48 to 110 kDa occurs after 30 to 45 min at 25 V in Towbin

Table 1. Description of the steps involved in the serodiagnosis of CBPP using the immunoblotting test (IBT)

Steps	Methods	Technical notes	Quality control
Antigen preparation	Preculture, in mycoplasma broth medium (Poumarat <i>et al.</i> , 1991), of the B103 strain (isolated in Portugal in 1986 from bovine lung tissue, Gonçalves <i>et al.</i> , 1998)	Strain B103 is available from the LNIV (Laboratório Nacional de Investigação Veterinária, Lisbon, Portugal) and from ANSES, Lyon Laboratory (UMR Ruminant Mycoplasmoses, 31 avenue Tony Garnier, 69364 Lyon cedex 07)	
	Seeded at 1:100 in 150 mL of mycoplasma broth medium. Incubation at 37°C, 5% CO ₂ , 48 to 66 h		Absence of mycoplasma contamination checked by "colony blotting" with a MmmSC-specific antibody
	Centrifugation at 12,000 xg, 30 min at 4°C. Mycoplasma pellets are washed three times in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4)		
	The pellet is resuspended with 1 mL of PBS and homogenised by repeated pipetting with a thin tip needle. The mycoplasma concentrate is stored at -20°C up to one year in 50 µL aliquots		The presence of the 110, 98, 95, 60-62 and 48 kDa proteins is checked by IBT with positive and negative reference sera ⁽¹⁾
Production of antigen strips	1 volume of the antigen diluted to 1:2 in distilled water is mixed with 1 volume of Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min	The denaturation buffer affects the quality of protein separation. Any buffer other than Laemmli buffer must be checked by IBT	
	Separation by electrophoresis on a Bio-Rad Mini-Protean TGX Any kD gel (Buffer: 2.5 mM Tris, 19.2 mM glycine, 0.01% SDS, pH 8.3) or on an Invitrogen NUPAGE 7% Tris-Acetate gel (NUPAGE buffer, Tris-acetate SDS), according to the manufacturer's recommendations	Electrophoresis is stopped when the 40 kDa protein of the Invitrogen NOVEX Sharp protein standard reaches the bottom of the gel	
	The gel, the nitrocellulose membrane (0.45 µm) and the filter papers (extra-thick filter papers, Bio-Rad, reference 170-3969) are allowed to equilibrate in Towbin buffer (25 mM Tris, 192 mM glycine) for 5 min at room temperature		
	Semi-dry transfer in Towbin buffer for 30 to 45 min at 25 V with the Trans-Blot SD or the Trans-Blot Turbo system from Bio-Rad		The quality of the transfer is checked by staining the membrane with R-RPOB from Sigma-Aldrich (according to the manufacturer's recommendations). The membrane is destained with 10 mM EDTA, pH 8.0 and rinsed twice with distilled water
	The membrane is blocked with 40 mL of blocking solution per membrane for 2 h at room temperature Blocking solution: 50 g/L dry skim milk, 75 g/L glycine, 10 g/L egg albumin, stored at -20°C up to 6 months		Batches of blocking buffer are tested by IBT with positive and negative reference sera
	The membrane is washed three times at room temperature for 15 min with 40 mL of TBS, 0.1% Tween 20 and once for 15 min with 40 mL TBS. TBS: 20 mM Tris, 500 mM NaCl pH 7.4	If the membrane is not washed, the intensity of the reaction will be reduced	
The membrane is cut into antigen strips (one membrane makes one batch of strips). The strips are dried at room temperature and then stored in an air-tight tube at -20°C up to one year		Two strips per batch are tested by IBT with positive and negative reference sera	

(1) The positive reference sera used are the 511-49 and 511-56 sera from the Abdo *et al.* (1998) study. The negative reference sera come from French cattle that have tested negative for CBPP using competitive ELISA and IBT



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Table 1. Description of the steps involved in the serodiagnosis of CBPP using the immunoblotting test (IBT) (following)

Steps	Methods	Technical notes	Quality control
Serodiagnosis by IBT	For each tested serum, an antigen strip is immersed in 1 mL of dilution solution and 200 µL of serum and then incubated for 2 h at room temperature with gentle shaking. Dilution solution: 0.1% dry skim milk (Bio-Rad "blotting-grade blocker" reference 170-6404), 0.1% egg albumin, stored at 20°C up to 6 months		Batches of dilution solution are tested by IBT with positive and negative reference sera. Positive and negative reference sera are used as controls for each batch of strips
	The strips are washed three times at room temperature for 15 min in TBS, 0.1% Tween 20 and once for 15 min in TBS		
	The secondary antibody is diluted in dilution solution, 1 mL is added to each strip and incubated for 2 h at room temperature with gentle shaking		The dilution of the secondary antibody is determined during the test by IBT with positive and negative reference sera when each new batch is received
	The secondary antibody: immunoaffinity-purified peroxidase-conjugated anti-bovine IgG (Sigma-Aldrich reference A5295)		
	The strips are washed three times at room temperature for 15 min in TBS, 0.1% Tween 20 and once for 15 min in TBS		
	During the last wash, the substrate is prepared (30 mg of 4-chloro-1-naphthol dissolved in 10 mL methanol joined to 50 mL PBS and 30 µL hydrogen peroxide)		Batches of 4-chloro-1-naphthol are tested on dot blots with dilutions of secondary antibodies to assess the reaction and check for the presence of any non-specific precipitation
	The strips are incubated with 2 mL of substrate until bands start to darken. The reaction is stopped by washing the strips in distilled water	The time it takes for the bands to darken is based on the positive and negative controls	
	The results are interpreted by comparing the lane containing the tested serum with those of the positive and negative controls		

buffer with the Trans-Blot SD or the Trans-Blot Turbo transfer system (Bio-Rad) (Table 1). When the filter papers, gels and membranes are assembled, bubbles or gel debris can hinder transfer resulting in the absence of one or more proteins from a lane in the membrane (this corresponds to an antigen strip). Since batches are tested on two randomly chosen strips, a transfer problem on one lane may go unnoticed and cause false negative or uninterpretable results, thereby extending analysis time. The homogeneity and the quality of the protein transfer must therefore be checked by using a reversible staining procedure that does not interfere with the IBT, such as the R-PROB reagent sold by Sigma-Aldrich (MacPhee, 2010). This step also allows the lanes to be marked for cutting the membrane into strips and for tracing the migration fronts to align and compare strips and therefore minimise edge effects. The membranes are then blocked (Table 1) and cut into antigenic strips.

By using ready-made gels and semi-dry electrotransfers, only 5 h are needed to prepare the antigen strips from the frozen protein extract. The number of strips produced ranges from 20 to 50, depending on the system used. The strips are dried (nitrocellulose membranes must be used, because they are easier to rehydrate compared to polyvinylidene fluoride membranes) and stored at -20°C for up to one year in an air-tight tube. Using reagents prepared this way, IBT serodiagnosis can be completed in 6 h.

Serodiagnosis by IBT

Each serum to be tested is diluted to 1:6 in a solution the composition of which (Table 1) influences the intensity of the

reaction and the background noise. Store-bought dry skim milk, commonly used as a blocking agent in western blotting (MacPhee, 2010) is not recommended due to the great variability among batches and suppliers and also due to the high background noise that occurs with certain sera, hindering interpretation (Figure 1B). The skim milk sold by Bio-Rad keeps background noise down (Figure 1C). Any new batch of skim milk must be assessed beforehand by running the IBT with positive and negative reference sera.

The prescribed secondary antibodies are immunoaffinity-purified anti-bovine IgG (Sigma-Aldrich) to ensure detection specificity. Upon reception, each new batch of antibody must be tested to determine the appropriate dilution and ensure similar sensitivity among antibody batches.

To read the IBT results, it is essential that the position of the 110, 98, 95, 60-62 and 48 kDa bands always be identified against a positive control serum for which the IBT profile is well characterised (reference serum, Table 1). The identification of the bands of interest is also facilitated by using several size standards with different molecular masses (Sagedi *et al.*, 2003) and a negative control serum. Then, the interpretation of the results is done by comparing the profiles of the controls against those from the tested sera. The interpretation of IBTs can sometimes be problematic when other bands appear near the MmmSC specific bands, since an edge effect can cause a slight shift in band position. In extreme cases, the strip can be cut in two lengthwise and one strip can be revealed with the positive serum and the other with the ambiguous serum. Joining the two halves of the strip then allows for precise comparison of the test serum profile with that of the positive control.



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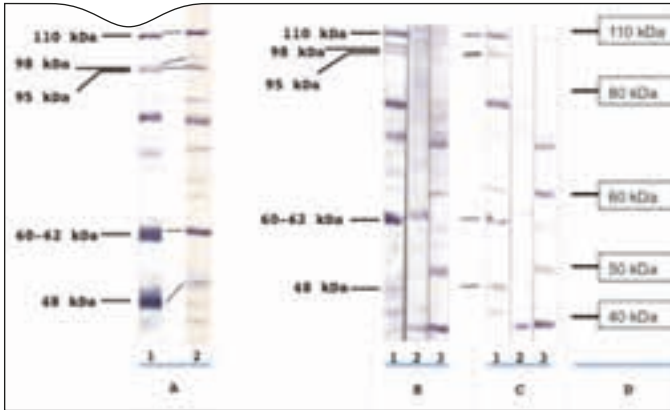


Figure 1. Immunoblotting test for CBPP serodiagnosis: influence of the type of gel and the serum dilution solution used on the obtained profiles.

A : profile of a positive serum on a Bio-Rad Mini-Protean TGX Any kD gel (lane 1) or an Invitrogen NUPAGE 7% Tris-Acetate gel (lane 2),

B and C: tested sera were diluted in a dilution solution made up with store-bought powdered skim milk (B) or Bio-Rad brand skim milk (C). Lane 1 contains the positive serum and lanes 2 and 3 the negative sera.

D: position of proteins in the molecular mass standard (Novex sharp protein standard from Invitrogen).

In conclusion, carefully choosing the consumables (i.e. reagents and materials) and identifying the critical steps in IBT can significantly improve repeatability and should enhance reproducibility, the main weakness of the IBT method as revealed in the last ILPT in 2009. IBT is regularly performed in our laboratory to test suspicious or positive sera, which occur frequently for the CF test and occasionally for the competitive ELISA.

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