Short Communication

Development of a rapid immunobinding test for Mycoplasma bovis cultural isolates in the genital tract of heifers

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ABSTRACT

An immunobinding test was developed on nitrocellulose paper with monoclonal antibody to diagnose Mycoplasma bovis in cultural isolates from the genital tract of artificially-infected heifers; polymerase chain reaction was used as the gold standard. The sensitivity of the immunobinding test was $5 \times 10^3$ colony-forming units, and was highly specific since there were no cross-reactions with other strains of Mycoplasma or Acholeplasma. Epidemiological sensitivity was 96.43%, epidemiological specificity 100.00%, positive predictive values 100.00%, and negative predictive values were 100.00%. There were no significant differences in diagnostic validity values between the immunobinding test and the PCR test ($P>0.05$). Thus, the immunobinding test is a reliable and alternative laboratory diagnostic technique for the M. bovis infection of the reproductive tract in female cattle under experimental conditions; however, it should be field tested in order to entirely evaluate the diagnostic validity of the procedure.

Key words: Immunobinding test, Mycoplasma bovis, Genital tract.

RIASSUNTO

Sviluppo di un immunobinding test rapido per isolati di Mycoplasma bovis nell’apparato genitale di manze

È stato sviluppato un immunobinding test su carta nitrocellulosa con anticorpi monoclonali al fine di diagnosticare Mycoplasma bovis in isolati di coltura provenienti dal tratto genitale di manze infettate artifi-
cialmente; è stata utilizzata la reazione a catena della polimerasi (PCR) come gold standard. La sensibilità dell’immunobinding test è stata di $5 \times 10^3$ CFU, unità formanti colonie; il test si è rilevato inoltre altamente specifico in quanto non si sono verificate reazioni crociate con altri ceppi di Mycoplasma o Acholeplasma. La sensibilità epidemiologica è stata del 96,43%, la specificità epidemiologica del 100,00% e il valore predittivo negativo del 100,00%, come pure il valore predittivo positivo. Non si sono evidenziate differenze significative nella validità dei valori diagnostici tra l’immunobinding test e il test PCR ($p>0,05$). L’immunobinding test si è rivelato affidabile come tecnica diagnostica alternativa per le infezioni da M. Bovis dell’apparato genitale femminile in condizioni sperimentali, tuttavia dovrebbe essere testato in condizioni “di campo” al fine di valutare completamente la validità diagnostica della procedura.

Parole chiave: Test immunobinding, Mycoplasma bovis, Apparato genitale femminile.

Introduction

In cattle, Mycoplasma spp. induce inflammatory lesions in the reproductive tract and cause reproductive failures (Flores-Gutierrez et al., 2004), with a focus in the female genital tract from which mycoplasmas can spread to other anatomical sites or to the foetus (Stipkovits et al., 1993). Thus, there is a requirement for effective and rapid detection of asymptomatic carriers to control the dissemination process (McCormack, 1976).

The diagnosis of bovine mycoplasmas has depended upon microbiological culture, which is satisfactory but difficult to carry out and time-consuming (Sachse et al., 1993; Hayman and Hirst, 2003). Also, molecular tests have been used satisfactorily but they require expensive equipment (Infante et al., 2002).

The immunobinding assays are sensitive and specific tests that have been developed to identify mycoplasmas in milk samples and semen, and do not require sophisticated and expensive equipment (Infante et al., 2002; Flores-Gutierrez et al., 2004). Thus, the objective of this study was to evaluate an immunobinding test (IBT) on nitrocellulose paper (NCP) with monoclonal antibodies (mAb) for detecting M. bovis from samples of artificially infected genital tracts of heifers.

Material and methods

Seven different strains of M. bovis, and single strains of M. bovigenitalium, M. bovicoli, M. bovirhinus, M. arginini, M. bovigenitalium, M. alkalescens, M. californicum, A. axanthum, A. modicum, A. laidlawii and A. granularum, isolated from field cases of cow-mastitis, growth in Hayflick broth medium and confirmed by immunoperoxidase test (Infante Martínez et al., 2004), were used to determine the specificity of the mAb used in this study. These cultures were washed four times with Tris-buffered saline solution (Sigma Chemical Company), pH 7.5 (TBS), adjusted to 1 mg of protein per millilitre and stored at -20°C prior to use.

The mAb used for developing the IBT, anti-M. bovis strain 201, was adjusted to contain 10 mg of globulin per millilitre. This solution was diluted 1:500 in a blocking solution of TBS (TBS+0.05% Tween 20+1.00% v/v bovine gelatin+0.5 mol NaCl [Sigma Chemical Company]), after which it was subjected to twelve 10-fold serial dilutions to establish the appropriate dilution to be used in the IBT.

The immunological sensitivity of the mAb was the lowest number of colony-forming units (CFU) of M. bovis that the assay could detect with very low or no noticeable background staining of NCP. The IBT was run with each of the serial dilutions of the mAb on six 10-fold serial dilutions of broth cultures of whole-cell M. bovis in TBS, ranging from 1:10 to 1:10⁶. The titre of the broth culture was determined by subculturing samples of the dilutions on agar for colony counts. The immuno-
logical specificity of the mAb was determined by carrying out the IBT on the Mycoplasma and Acholeplasma strains listed above.

The experimental samples were obtained from sixty heifers, 12 months, with a negative microbiological culture of vaginal swabs on modified Hayflick medium. Then, the heifers were inoculated in the uterus as follows: 30 heifers were inoculated with 10 mL of modified Hayflick medium containing \(1.5 \times 10^7\) CFU/mL of M. bovis strain 201 and diluted in 20 mL of TBS; 20 heifers were inoculated with 10 mL of modified Hayflick medium containing \(1.5 \times 10^7\) CFU/mL of M. bovigenitalium, and diluted in 20 mL of TBS, and 10 heifers were inoculated with 30 mL of sterile TBS. Similar inoculums have been reported for several experimental studies (Boothby et al., 1986; Peltier et al., 2007), and also higher inoculums than those reported here (Brown et al., 1996; Shkarupeta et al., 2004).

Three days after inoculation, vaginal swabs were deposited in a transport medium consisting of mycoplasma broth supplemented with thallium acetate, ampicillin (Sigma Chemical Company) and equine serum, and stored at 4°C. Once in the laboratory, the samples were incubated at 37°C in a 10% CO\(_2\) atmosphere for 36 hours to increase the number of CFU. The IBT reactions were carried out, at room temperature, on strips of 1x1cm of NCP (Applied Scientific Company), pore size of 0.45 µm with a capacity to concentrate 80 µg protein/cm\(^2\), on which 5 µl of the enriched antigen was spotted; this enriched antigen was the abovementioned vaginal-swabs culture.

The test procedures were the following. The strips were incubated for 10 min with 1.5 mL of blocking solution to fill the pores of the NCP and prevent non-specific reactions; the blocking solution also was used as a diluent for serum and conjugates. After this, 1.5 mL of each of the 10-fold serial dilutions of the mAb in blocking solution were incubated for 60 min for determining its correct work dilution. The following step consisted of the incubation for 30 min of biotinylated rabbit anti-goat IgG (H+L) (Bio-Rad Laboratories) diluted 1:1000 in blocking solution and subsequently with horse-radish peroxidase (Sigma Chemical Company) diluted 1:500 in blocking solution was incubated for 30 min.

The substrate consisted of a mixture of

Solution A: (10 mL cold methanol+30 mg 4-chloro-naphthol [Sigma Chemical Company]) and

Solution B: (50 mL TBS+30 µl H\(_2\)O\(_2\) 30% [Sigma Chemical Company]), which was prepared shortly before use. With 4 mL of this substrate, incubated for 10 min., a positive reaction was revealed as a dark-blue spot on the NCP; the presence or absence of this colour-reaction determined whether there was a positive or a negative result. Between each incubation period 1.5 mL of a washing solution (TBS with 0.05% Tween 20 [Sigma Chemical Company]), were used for 5 min., in order to eliminate the unbound conjugates and antibodies. Also, the reproducibility of the immunobinding test was evaluated by performing this test three times on each sample.

The gold standard was the polymerase chain reaction (PCR) described elsewhere (Chávez-González et al, 1995), which was carried out in the same enriched samples than the IBT. The PCR was used to calculate the diagnostic validity, i.e., epidemiological sensitivity (Se) (the absence of false-negative results), the epidemiological specificity (Spe) (the absence of false-positive results) and the positive predictive value (PV+) and negative predictive value (PV-) (the probability of a true diagnosis, no matter whether positive or negative) of the IBT at a 95% level of confidence. The results of both tests were statistically analysed with the Kappa test at the same level of confidence with the Episcope 2.0 software (Veterinary School, Zaragoza, Spain).
Results and discussion

The appropriate dilution for the mAb was the 1:1000 because with this the IBT could detect the lowest number of CFU with very little or no noticeable background staining of the NCP. The immunological sensitivity obtained for the IBT, evaluated using the enriched samples was 5x10³ CFU. Furthermore, this test was highly specific because it had no cross-reactions with the Mycoplasma and Acholeplasma strains used in this study. The reproducibility of the developed test was satisfactory, obtaining identical results when all the samples were analysed three times. In addition, the IBT was carried out in a total time of one hour and 59 min.

The IBT correctly identified 95% of the samples (three apparent false-negative results), while the PCR had a correct identification of samples on the order of 96.6% (two apparent false-negatives). However, in the samples identified as negative by PCR there was no growth of microorganisms on modified Hayflick medium.

In agreement with the values of Table 1, the values of Se, Spe, PV+ and PV- for the IBT were 96.429%, 100.00%, 100.00% and 96.970%, respectively. There were no significant differences in the results of the tests (P>0.05), since the Kappa value was 0.966.

The diagnosis of mycoplasmal infections in cattle has depended upon microbiological culture (Ruhnke et al., 1978), which is satisfactory but difficult to carry out and time-consuming (Sachse et al., 1993; Hayman and Hirst, 2003). In addition, molecular tests (Uhaa et al., 1990) have been used satisfactorily but they require expensive equipment (Infante et al., 2002).

Mycoplasmal infections in the reproductive tract of female cattle cause large economic losses (Sachse et al., 1993), mainly by reducing calving rates, prolonging calving-to-service intervals and calving-to-pregnancy intervals of affected cows (Eaglesome and Garcia, 1990; Uhaa et al., 1990; Stipkovits et al., 1993). In addition, M. bovis can be spread from the reproductive tract to other anatomical sites and to the foetus (Stipkovits et al., 1993). Therefore, it is important identify both, asymptomatic carriers and infected animals, in order to limit the spread of this infections into mycoplasma-free herds; this requires a sensible and specific diagnostic technique (Sachse et al., 1993).

Microbiological culture is a sensitive and specific method to identify Mycoplasma; however, immunological tests are faster than the former techniques (Heller et al., 1993). ELISA and PCR are sensitive and specific tests used for diagnosing M. bovis and some authors consider the sensitivity of immunobinding assays to be low when compared with them (Heller et al., 1993; Sachse et al., 1993; Pinnow et al., 2001). Neverthe-

### Table 1

Results of the polymerase chain reaction and the immunobinding test on nitrocellulose paper with monoclonal antibodies to diagnose Mycoplasma bovis in cultural isolates in the genital tract of heifers.

| Polymerase chain reaction (gold standard) | Positive | Negative | Total |
|-----------------------------------------|----------|----------|-------|
| Immunobinding assay                     |          |          |       |
| Positive                                | 27       | 0        | 27    |
| Negative                                | 1        | 32       | 33    |
| Total                                   | 28       | 32       | 60    |
less, other authors have reported that immunobinding assays and PCR are equally sensitive in the diagnosis of *M. bovis* infection (Hayman and Hirst, 2003).

In this study, the IBT had a lower sensitivity than the PCR; however, there were no significant differences between the results of the two tests. In addition, both tests have the same specificity, since there were no cross-reactions with mycoplasmas other than *M. bovis*. The values for epidemiological sensitivity and specificity of the IBT are considered as acceptable; because were higher than those reported for an ELISA, which obtained 80.6% and 94.9% for Se and Spe, respectively (Heller *et al.*, 1993).

**Conclusions**

Our conclusion is that the IBT constitutes a reliable and alternative diagnostic technique for the *M. bovis* infection of the reproductive tract in female cattle under experimental conditions. However, it should be field tested in order to entirely evaluate the diagnostic validity of the procedure (i.e. Se, Spe, PV+ and, PV-).

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