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# COMPARISON BETWEEN ENZYME-LINKED IMMUNOSORBENT ASSAY, DISC FILM INHIBITION AND COMPLEMENT FIXATION TESTS FOR THE DIAGNOSIS OF *MYCOPLASMA BOVIS*<sup>1</sup>

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**ABSTRACT** - A total of 1300 bovine field sera was tested by the Indirect ELISA, Complement Fixation (CF) and Disc Film Inhibition (DFIT) Tests, for the detection of *Mycoplasma bovis* infections by the determination of circulating antibodies in naturally infected cattle. ELISA showed the highest specificity (96%) for detection of antibody against *M. bovis*, when ELISA OD  $\geq$  0.900 was considered as positive, testing negative sera in DFIT, and considering CF titres of 1/40 as positive. For sensitivity evaluation, the highest sensitivity of ELISA (90%) was obtained considering ELISA OD  $\geq$  0.600 as positive, testing sera positive in DFIT, and considering CF titres of 1/40 as negative. The ELISA proved to be a sensitive and specific test for *Mycoplasma bovis* serodiagnosis. The immunoglobulin classes measured by each of the tests under study are discussed.

Index terms: mycoplasmosis, serodiagnosis, antibody detection.

## COMPARAÇÃO ENTRE OS TESTES ELISA, INIBIÇÃO DE FILMES EM DISCO E FIXAÇÃO DE COMPLEMENTO PARA O DIAGNÓSTICO DE *MYCOPLASMA BOVIS*

**RESUMO** - Um total de 1300 amostras de campo de soros bovinos foi testado pelos testes ELISA Indireto, Fixação de Complemento (CF) e Inibição de Filmes em Disco (DFIT) para a detecção de infecções por *Mycoplasma bovis*, pela determinação de anticorpos circulantes em gado naturalmente infectado. O ELISA mostrou a maior especificidade (96%) para detecção de anticorpos contra *M. bovis*, quando foi considerado ELISA OD  $\geq$  0.900 como positivo, testando-se soros negativos em DFIT, e considerando-se títulos de CF de 1/40 como positivos. A maior sensibilidade do ELISA (90%) foi obtida considerando-se ELISA OD  $\geq$  0.600 como positiva, testando-se soro positivo em DFIT e considerando-se títulos de CF de 1/40 como negativos. O ELISA provou ser um teste sensível e específico para o soro-diagnóstico de *Mycoplasma bovis*. Discutem-se as classes de imunoglobulinas detectadas em cada teste utilizado no estudo.

Termos para indexação: micoplasmoses, soro-diagnóstico, detecção de anticorpos.

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## INTRODUCTION

The Complement Fixation Test (CFT) was one of the first serological tests used for the diagnosis of mycoplasma infections, and was standardized in 1936 by Campbell & Turner, for the serodiagnosis of Contagious Bovine Pleuro-pneumonia

(CBPP). Since then, the CFT has been used for many years, in different laboratories, as one of the reference tests for serodiagnosis of CBPP as well as for other diseases caused by *Mycoplasma* species (Al-Aubaidi & Fabricant, 1971a; 1971b; Howard et al., 1977; Vincent et al., 1984), although it is a complex, laborious and time consuming test (Fischer et al., 1986).

Film Inhibition (FI) was first used as a serodiagnostic test by Ruhnke et al. (1976) to indicate whether the animal has had a past infection with *M. bovis*. In 1978 Thorns & Boughton described a Disc Film Inhibition Test (DIFT), and they showed that sera from naturally infected animals produced film inhibition, but sera prepared in the laboratory generally showed weak or no activity.

Attempts to use ELISA techniques for the diagnosis of *M. bovis* infections have increased since Boothby et al. (1981) first described its use for the detection of *M. bovis* specific IgG in bovine serum. However, the reports were obtained from experimentally infected animals (Boothby et al., 1982a; 1982b; Mills & Frey, 1982; Howard et al., 1983), and the ELISA was not employed for survey purposes nor to diagnose infected animals in naturally infected herds.

This paper describes the use of an Indirect ELISA, compared with CFT and DIFT, in a serological survey for the presence of antibodies in animals exposed to *M. bovis* in the field.

## MATERIALS AND METHODS

### Sources of sera:

The bovine field serum samples tested were sent to the Mycoplasma Section at the Central Veterinary Laboratory, Weybridge, Surrey, England, for serodiagnosis of *Mycoplasma* infections. They were collected from animals suspected of suffering from diseases caused by *Mycoplasma* spp., or from animals to be exported from or imported to the United Kingdom. The serum samples were kept at -20°C until used. Each serum was identified by a number, in order of arrival at the Laboratory. After being tested, the serum samples were again stored frozen at -20°C.

### Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect ELISA used for *M. bovis* antibody de-

tection was one standardized by Liberal (1988) using *M. bovis* sonicated antigen, PVC flexible plates, Horse Radish Peroxidase rabbit anti-bovine conjugate, OPD substrate and sulphuric acid (to stop the enzymatic reaction). One hundred µl of reagents (at working dilution) were distributed in each of the 96 flat bottomed wells of a plate, except for sulphuric acid, used in 30µl amount. A standard plate lay-out was used to allow a uniform reading of each plate. Positive and negative control sera for *M. bovis* were used in each microtitre plate, as well as a background control. The ELISA reader was blanked on air, and the results expressed as optical density (OD) measured at 490 nm. A computer program was set up to read all plates according to the plate lay-out, giving a Crude OD, a Control OD and a Net OD, for each pair of serum samples tested. From that the average Net OD was calculated and the values adjusted to the previously established value for the Positive control serum (OD=1400).

### Disc Film Inhibition Test (DIFT)

All bovine field sera tested with ELISA were also tested using the DIFT described by Thorns & Boughton (1978). The field sera were used to impregnate sterile filter paper discs, and a *M. bovis* reference strain (5B 252 Central Veterinary Laboratory Culture Collection) was used throughout the experiment. The DIFT results were read visually as positive and negative.

### Complement Fixation Test (CFT)

The CFT was carried out in microtitreplates following essentially the recommendations of Thorns & Boughton (1978), using CFT diluent, and sheep red blood cells in a 1.5% concentration sensitised with 0.25% sheep haemolysin, as the haemolytic system, and freeze dried guinea pig complement in 20% Richardson's preservative.

## RESULTS AND DISCUSSION

A total of 1300 bovine field sera was tested by the Indirect ELISA, Complement Fixation and Disc Film Inhibition Tests, for the detection of *M. bovis* infections by the determination of circulating antibodies in naturally infected cattle.

Since for each of the three tests the results obtained are expressed in a different way, it was necessary to rearrange the results for interpretation and comparison as follows:

ELISA: Positive ELISA = net OD higher than

0.900

Inconclusive ELISA = net OD between 0.600 and 0.900

Negative ELISA = net OD lower than 0.600

CFT: Positive CFT = titres 1/80 and 1/160

Inconclusive CFT = titres 1/40

Negative CFT = titre of 1/20 or less

DIFT: Positive DIFT = inhibition of growth and/or film production around the disc

Negative DIFT = growth and film production around the disc

### Comparison of the positive DIFT group:

#### 1. With ELISA

A total of 172 serum samples gave positive results in DIFT. From those, 48.8% were positive ELISA; 10.5% were inconclusive ELISA; and 40.7% were negative ELISA. Considering OD values between 0.600 and 0.900 as positive, the percentage of positive ELISA result was 59.3.

#### 2. With CFT

The CFT results were: negative CFT = 50.5%; 1/10 = 5.8%; 1/20 = 6.4%; 1/40 = 14.0%; 1/80 = 14.0%; and 1/160 = 9.3%. Considering positive CF titres as 1/80 and 1/160, the percentage of positive CF results were 23.3. If titres of 1/40 were also considered as positive, the total of CF positive results were 37.3%.

### Comparison of the negative DFIT group:

#### 1. With ELISA

A total of 1128 serum samples gave negative results in DFIT. The results obtained using ELISA were: 90.6% of negative ELISA; 3.6% of inconclusive ELISA; and 5.8% of positive ELISA. Considering OD values between 0.600 and 0.900 as negative, the percentage of negative ELISA result was 94.2%.

#### 2. With CFT

For CFT the results obtained were: negative CFT = 61.2% ; 1/10 = 8.6; 1/20 = 9.3%; 1/40 =

11.0%; 1/80 = 5.2%; and 1/160 = 4.7%. Considering negative CFT as 1/20 and lower, the percentage of negative results was 79.1%; if 1/40 was also considered as a negative result, the final percentage of negative was 90.1.

When ELISA OD  $\geq$  0.900 was considered as positive, the ELISA gave less sensitive (49% against 59%) but more specific (94% against 91%) results than when ELISA OD  $\geq$  0.600 was considered as positive: the chi-squared test gave values of  $p > 0.05$  and  $p > 0.01$  respectively; the coefficients of association (0.88 against 0.87) and colligation (0.60 against 0.58) between tests being higher with OD  $\geq$  0.900.

When a CF titre of 1/40 was considered as positive, the DIFT gave less sensitive (21% against 26%) but slightly more specific (89% against 88%) results than when CF titre of 1/40 was considered as negative with smaller coefficients of association (0.38 against 0.47) and colligation (0.20 against 0.25) between tests and chi-squared test values of  $p < 0.001$  and  $p > 0.05$  respectively.

Analysing the data obtained in this comparison, it seems that DIFT positive and negative results correlate better with ELISA than with CFT results.

### Comparison of ELISA and CFT using the positive DIFT sera:

The results are presented in Tables 1 and 2, and were analysed as ELISA negative, inconclusive, and positive as follows:

**ELISA negative:** negative CFT = 75.7%; 1/10 = 7.2%; 1/20 = 5.7%; 1/80 = 4.3%; 1/160 = 1.4%. Considering as CF negative titres of 1/20 and lower, the percentage of negative CF results was 88.6%. If 1/40 CF titre was included as negative, the percentage was 94.3%.

**ELISA inconclusive:** negative CFT = 33.4%; 1/10 = 11.1%; 1/20 = 11.1% 1/40 = 11.1%; 1/80 = 11.1%; 1/160 = 22.2%.

**ELISA positive:** negative CFT = 33.3%; 1/10 = 3.6%; 1/20 = 6.0%; 1/40 = 21.4%; 1/80 = 22.6%; 1/160 = 13.1%. Considering CF titres of 1/80 and 1/160 as positive, the percentage of positive CFT results was 35.7%. If a titre of 1/40

**TABLE 1. Comparison between ELISA and CFT in bovine serum samples with positive CFT and DFIT results.**

Optical density (OD)	CFT Titre			Total	
	1/40	1/80	1/160	1/80+1/160	1/40,1/80,1/160
0.001 - 0.600	4	3	1	4	8
0.601 - 0.900	2	2	4	6	8
0.901 - 1.900	18	19	11	30	48
Total	24	24	16	40	64

Considering CFT 1/40 as positive

**TABLE 2. Comparison between ELISA and CFT in bovine serum samples with negative CFT and positive DFIT results.**

Optical density (OD)	CFT Titre				Total	
	Neg	1/10	1/20	1/40	Neg, 1/10, 1/20	Neg, 1/10, 1/20, 1/40
0.001-0.600	53	5	4	4	62	66
0.601-0.900	6	2	2	2	10	12
0.901-1.900	28	3	5	18	36	54
Total	87	10	11	24	108	132

Considering CFT 1/40 as negative

was also considered as positive, 51.7% of the samples were positive in CFT.

If the ELISA inconclusive group was regarded as positive, the results obtained for CFT were: negative CFT = 33.3%; 1/10 = 4.9%; 1/20 = 6.9%; 1/40 = 19.6%; 1/80 = 20.6%; 1/160 = 14.7%. When a positive result was taken as 1/80 and 1/160 titres, the percentage of positive was 35.3%, including 1/40 as positive, the positive CFT results represented 54.9% of the samples.

Considering CF titre of 1/40 as positive and ELISA OD  $\geq$  0.900 also as positive, the ELISA gave less sensitive (75% against 87%) but more specific (67% against 57%) results than when ELISA OD  $\geq$  0.600 was considered as positive, with smaller coefficients of association (0.71 against 0.81) and colligation (0.42 against 0.51) between tests and chi-squared test values of  $p > 0.01$  and  $p < 0.001$  respectively.

Considering CF titre of 1/40 as negative and ELISA OD  $\geq$  0.900 as positive, the ELISA gave

less sensitive (75% against 90%) but more specific (59% against 50%) results than when ELISA OD  $\geq$  0.600 was considered as positive; with smaller coefficients of association (0.63 against 0.80) and colligation (0.35 against 0.50) between tests and chi-squared test values of  $p < 0.001$  in both situations.

In summary, from the 172 bovine serum samples that showed positive DIFT results for *M. bovis*, 59.3% were positive in ELISA and 37.3% positive in CFT, considering ELISA positive results as OD higher than 0.600 and CFT positive results with titres of 1/40 and higher. Using these parameters, 54.9% of positive CFT results were correlated with positive ELISA results. With this set of data, ELISA was the most sensitive test for *M. bovis* antibody detection.

#### Comparison of ELISA and CFT using the negative DFIT sera:

The results are shown in Tables 3 and 4, and

**TABLE 3. Comparison between ELISA and CFT in bovine serum samples with negative CFT and DFIT results.**

Optical density (OD)	CFT Titre				Total		
	Neg	1/10	1/20	1/40	Neg, 1/10, 1/20	Neg, 1/10, 1/20, 1/40	
0.001-0.600	655	89	91	101	835		936
0.601-0.900	18	4	3	6	25		31
0.901-1.900	17	4	11	17	32		49
Total	690	97	105	124	892		1016

Considering CFT 1/40 as negative

**TABLE 4. Comparison between ELISA and CFT in bovine serum samples with positive CFT and negative DFIT results.**

Optical density (OD)	CFT Titre			Total	
	1/40	1/80	1/160	1/80+1/160	1/40, 1/80, 1/160
0.001-0.600	101	51	35	86	187
0.601-0.900	6	2	8	10	16
0.901-1.900	17	6	10	16	33
Total	124	59	53	112	236

Considering CFT 1/40 as positive

were again analysed as ELISA negative, inconclusive and positive, as follows:

**ELISA negative:** negative CFT = 64.1%; 1/10 = 8.7%; 1/20 = 8.9%; 1/40 = 9.9%; 1/80 = 5.0%; 1/160 = 3.4%. Considering as CF negative titres of 1/20 and lower, 81.7% of the CF results were negative. This percentage increased to 91.6% if 1/40 titre was also considered as negative.

**ELISA inconclusive:** negative CFT = 43.9%; 1/10 = 9.7%; 1/20 = 7.3%; 1/40 = 14.7%; 1/80 = 4.9%; 1/160 = 19.5%.

**ELISA positive:** negative CFT = 26.2%; 1/10 = 6.1%; 1/20 = 16.9%; 1/40 = 26.2%; 1/80 = 9.2%; 1/160 = 15.4%. Considering CF titres of 1/80 and 1/160 as positive, 24.6% of samples gave positive CF results. If a titre of 1/40 was also considered as positive, 50.8% of samples were CF positive.

If the ELISA inconclusive group was regarded as negative, the results obtained in comparison with CFT were: negative CFT = 63.3%; 1/10 =

8.8%; 1/20 = 8.8%; 1/40 = 10.1%; 1/80 = 5.0%; 1/160 = 4.0%. If a negative CF result was taken as titres of 1/20 and less, the percentage of negative CF results was 80.9%. Considering 1/40 as also negative, 91.0% of samples gave a negative result.

Considering CF titre of 1/40 as positive and ELISA OD  $\geq$  0.900 also as positive, the ELISA gave less sensitive (14% against 21%) but more specific (96% against 94%) results than when ELISA OD  $\geq$  0.600 was considered as positive, with higher coefficients of association (0.63 against 0.59) and colligation (0.35 against 0.32) between tests and chi-squared test values of  $p < 0.001$  respectively.

Considering CF titres of 1/40 as negative and ELISA OD  $\geq$  0.900 as positive, the ELISA gave less sensitive (14% against 23%) but more specific (95% against 92%) results than when ELISA OD  $\geq$  0.600 was considered as positive, with smaller coefficients of association (0.53 against

0.56) and colligation (0.29 against 0.31) between tests and chi-squared test values of  $p < 0.001$  and  $p > 0.05$  respectively.

In summary, from 1128 bovine serum samples with a negative DFIT result for *M. bovis*, 94.2% were negative in ELISA and 90.1% negative in CFT, when ELISA OD values between 0.600 and 0.900 were considered as negative and 1/40 CF titre and lower were also considered as negative. Thus, 91.0% of negative result in CFT were correlated with negative ELISA results. In this set of data again ELISA was the most sensitive test for *M. bovis* antibody detection, as in 49.2% of ELISA positive results, the CFT titres ranging from negative to 1/20, and 5.8% of samples that gave positive result in ELISA were negative in DFIT.

#### Comparison of ELISA and CFT results (independent of DFIT results):

Grouping the data obtained according to CFT results, the following observations were made:

- Considering CF titres of 1/40 as positive and ELISA OD  $\geq 0.900$  also as positive, the ELISA gave less sensitive (27% against 35%) but more specific (93% against 90%) results than when ELISA OD  $\geq 0.600$  was considered as positive, with higher coefficients of association (0.67 against 0.65) and colligation (0.38 against 0.37) between tests and chi-squared tests values of  $p < 0.001$  in both situations.

- Considering CF titre of 1/40 as negative and ELISA OD  $\geq 0.900$  as positive, the ELISA gave less sensitive (30% against 41%) but more specific (91% against 87%) results than when ELISA OD  $\geq 0.600$  was considered as positive, with smaller coefficients of association (0.63 against 0.65) and colligation (0.35 against 0.37) between tests and chi-squared test values of  $p > 0.001$  respectively.

#### General comparison between ELISA, CFT and DFIT results:

The results obtained from all 1300 sera tested, considering an inconclusive group in ELISA and CFT, are shown in Tables 5 to 7.

In a general comparison, ELISA showed the highest specificity (96%) for detection of antibody

**TABLE 5. Results obtained from sera positive in DFIT.**

Test	Positive	Inconclusive	Negative	Total
ELISA	84	18	70	172
CFT	40	24	108	172
Total	124	42	178	344

with chi-squared = 24.6 ( $p < 0.001$ )

**TABLE 6. Results obtained from sera negative in DFIT.**

Test	Positive	Inconclusive	Negative	Total
ELISA	65	41	1022	1128
CFT	112	124	892	1128
Total	117	165	1914	2256

with chi-squared = 63.06 ( $p < 0.001$ )

**TABLE 7. Results obtained from 1300 sera samples tested.**

Test	Positive	Inconclusive	Negative	Total
ELISA	149	59	1092	1300
CFT	152	148	1000	1300
Total	201	207	2092	2600

with chi-squared = 42.33 ( $p < 0.001$ )

against *M. bovis*, when ELISA OD  $\geq 0.900$  was considered as positive, testing sera negative in DFIT and considering CF titres of 1/40 as positive. The second highest specificity of ELISA (95%) was obtained again with ELISA OD  $\geq 0.900$  considered as positive, testing sera negative in DFIT and considering CF titres of 1/40 as negative. For sensitivity evaluation, the highest sensitivity of ELISA (90%) was obtained considering ELISA OD  $\geq 0.600$  as positive, testing sera positive in DFIT and considering CF titres of 1/40 as negative; the second highest sensitivity of ELISA (87%) was obtained with ELISA OD  $\geq 0.600$  considered as positive, testing sera positive in DFIT and CF titres of 1/40 as positive.

#### DISCUSSION

Comparisons have been made between ELISA and other serological tests to identify the most

sensitive and specific test for detection of antibodies against mycoplasmas. ELISA was found to be superior to CFT when comparing sensitivity, duration of antibody response, and technical requirements (Horowitz & Cassell, 1978; Onoviran & Taylor-Robinson, 1979; Armstrong et al., 1983; Piffer et al., 1984; Fischer et al., 1986). These reports are in agreement with the results obtained in this research, in which ELISA was superior to CFT and to DFIT. No report comparing DFIT with ELISA was found in the bibliographic review.

CFT is a time-consuming test, and some sera show anticomplementary activity when tested for mycoplasma antibody detection. DFIT appears to be sensitive and specific, although the test is greatly influenced by the *M. bovis* strain used to seed the plates. According to Thorns & Boughton (1978) there was considerable difference in the zones produced by the same serum on lawns of different strains and in the degree of dilution before inhibition was extinguished.

It is important to remember that to perform this comparison a question arises as to the class of antibodies measured by the different serological methods, since this indirect ELISA was developed to detect IgG class only; in CFT only IgM and some IgG subclass are involved (Busolo et al., 1983); and in DFIT only IgG2 class antibodies are detected (Thorns, 1978).

The reports published so far on the use of ELISA for detection of *M. bovis* specific IgG in bovine serum have addversed results obtained from animals experimentally infected, vaccinated or challenged with *M. bovis*, when only a small number of animals was evaluated (Boothby et al. 1981; Boothby et al. 1982a; 1982b; Howard et al. 1983). In this study, the indirect ELISA was evaluated using 1300 bovine field sera and proved to be a sensitive and specific test for *Mycoplasma bovis* serodiagnosis.

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