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The Evaluation of Humoral Immune Response to Contagious Bovine Pleuropneumonia in Cattle with Respiratory Disorders by Western Blot Technique, Competitive Enzyme Linked Immunosorbent Assay and Complement Fixation Test

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Abstract

In this study 253 serum samples from cattle showing respiratory disorders were tested by confirmatory Western Immunoblotting test (IBT), complement fixation test (CFT) and competitive enzyme linked immunosorbent assay (c-ELISA). Two (0.8%) out of 253 serum samples were found to be positive and two were (0.8%) doubtful by CFT while 7 (2.8%) serum samples were found as positive and 25 (9.9%) of them were found to be doubtful by c-ELISA. On the other hand, a core profile of antigenic bands needed to be identified at 110, 98, 95, 62/60 and 48 kDa was not detected in any of the test serum sample. Immunoblot analysis some of serum samples displayed the number of bands between one or three and some of them were highly faint. In order to detect similarities between MmmSc strains related to their electrophoretic profiles, whole cell proteins of *Mycoplasma* species isolated from cattle and MmmSC reference strains (*Mycoplasma mycoides subsp. mycoides* SC Botswana (African strain), *Mycoplasma mycoides subsp. mycoides* SC PG1 (reference strain), *Mycoplasma mycoides subsp. mycoides* SC V5 (Australian strain), *M. bovigenitalium, M. bovirhinis, M. capricolum subsp. capricolum*, *M.canadense, M.alkalescens and M. bovis*) were analysed by sodium dodecyl sulphate polyacrilamide gel elektrophoresis (SDS-PAGE). The result of this analysis indicated that antigens of 220, 78, 82, 68, 24 kDa were common among all tested strains. According to these results, it was thought that positive results by both CFT and c-ELISA, also non specific band patterns observed by IBT might be caused by a cross reaction between tested *Mycoplasma* strains. As conclusion, it is recommended that all the positive results by CFT and c-ELISA be confirmed by IBT.

Key Words: ELISA, immunoblotting test, serology

Solunum Bozukluğu olan Sığırlarda Bulaşıcı Sığır Pleuropnömonisine Karşı Oluşan Hümoral İmmün Yanıtın Western Blot Tekniği, Kompleman fiksasyon testi ve Kompetatif Enzyme Linked İmmunosorbent Assay Testi ile Değerlendirilmesi

Öz

Bu çalışmada, solunum bozuklukları gösteren sığırlardan alınan 253 serum numunesi, doğrulayıcı olarak Western Immunoblotting test (IBT), Kompleman fiksasyon testi (CFT) ve kompetatif enzyme linked immunosorbent assay (c-ELISA) ile test edildi. CFT ile 253 serum örneğinden ikisi (%0,8) pozitif, ikisi (%0,8) şüpheli, c-ELISA ile 7 (%2,8) serum numunesi pozitif ve 25'i (%9,9) şüpheli bulundu. Öte yandan, 110, 98, 95, 62/60 ve 48 kDa' olarak tanımlanması gereken antijenik bantlar, serum örneklerinin hiçbirinde tespit edilmedi. Bazı serum numunelerinin İmmünoblot analizi sonucunda bir veya üç arasında bant görüldü ve bu bantların bazıları oldukça soluktu.
MmmSc suşları arasındaki elektroforetik profiller ile ilgili benzerlikleri tespit etmek için sığırlardan izole edilen *Mycoplasma* türlerinin tam hücre proteinleri ve MmmSC referans suşları (*Mycoplasma mycoides subsp. mycoides* SC Botswana (Afrika suşu), *Mycoplasma mycoides subsp. mycoides* SC PG1 (referans suş), *Mycoplasma mycoides subsp. mycoides* SC V5 (Australian strain), *M. bovigenitalium, M. bovirhinis, M. capricolum subsp. capricolum, M.canadense, M.alkalescens and M. bovis*). sodium dodecyl sulphate polyacrilamide gel elektroforezi (SDS-PAGE) ile analiz edildi.
Bu analizin sonucu, 220, 78, 82, 68, 24 kDa antijenlerinin test edilen tüm suşlar arasında yaygın olduğunu gösterdi. Bu sonuçlara göre, CFT ve c-ELISA pozitif sonuçlarının ve IBT ile gözlemlenen spesifik olmayan bant örneklerinin, test edilen mikoplazma suşları arasındaki çapraz reaksiyonlardan kaynaklanabileceği düşünüldü. CFT ve c-ELISA ile elde edilen tüm pozitif sonuçların IBT testi ile doğrulanması gerektiği sonucuna varıldı.

Anahtar Kelimeler: ELISA, immunoblotting test, seroloji

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp *mycoides SC,* small colony (MmmSC) is an important contagious respiratory disease of cattle. The disease is charactherized by fever, anorexia, cough, dyspnoea, polypnoea and nasal charges associated with pneumonia and sero-fibrinous pleurisy. The disease occurs

in several forms changing from hyperacute to chronic and subclinical forms (1-4).

Serological analysis is the most important diagnostic tool for the control of CBPP. However, there is no single serological test capable of detecting all animals affected by CBPP under field conditions of infection. Complement fixation test (CFT) and competitive ELISA (c-ELISA) are highly specific tests but have been shown to be relatively sensitive in the very early and chronic stages of infection. Although CFT is a widely used official test approved by OIE, Immunoblotting test (IBT) is considered to be of diagnostic value showing a higher sensitivity and specificity and it is recommended to be used primarily as a confirmatory test. The presence of the core Ig G immunoblot profile of five specific antigen bands with determined molecular weights of 110, 98, 95, 62/60 and 48 kDa are considered positive for the infection (2, 5-7). IBT also demonstrates common antigenic bands with molecular weights of 85, 80, 72, 44 ve 39 kDa that might be detected between MmmSC strains (8).

Based on the literature reviewed in Turkey. Erdağ and Türkaslan, (1994) analyzed 750 serum samples collected from various locations of Turkey by CFT and agar gel diffusion tests and they failed to detect any positive sample. In another study, serum samples collected from 945 cattle from four abottoirs in Turkey were tested by CFT and cELISA. Four of the serum samples were found as positive by cELISA (0.4 %) while two of them were positive by CFT (0.2 %) (10).

In this study, a total of 253 serum samples from cattle experienced respiratory findings were tested by CFT, c-ELISA and IBT. It was aimed to evaluate and analyse host humoral immune response in relation to the electrophoretic profile of MmmSc antigens. It was also aimed to make observation to some extent about the status of the disease in our country.

MATERIALS AND METHODS

Serum Samples

A total of 253 serum samples which are sent to our laboratory for the diagnosis of CBPP from 18 different provinces and collected from the cattle showing respiratory symptoms were used in the study.

Reference Strains

Strains were obtained from Animal Plant Health Agency (APHA) and Pendik Veterinary Control Institute (PVCRI) culture collection.

Standard Positive and Negative Sera

Control sera were kindly obtained from Instituto Zooprofilatico Sperimentale (IZS) Terramo. A titer of 1:320 of positive serum was used in CFT and 1:200 in IBT.

MmmSC Antigen

A dilution of 1:70 standard antigen used in CFT was kindly obtained from IZS.

Eaton's Broth Media

The media was prepared according to the procedure decribed elsewhere (11).

Complement Fixation Test

CFT was carried out in microplate format according to the procedure recommended by OIE (2).

Competitive ELISA

Institut Pourquier CIRAD "CBPP serum competition ELISA" kit (Version: P05410/01) was used acccording to the instructions of manufacturer.

Antigen Preparation for Immunoblotting

For antigen preperation, 1 ml *Mmm* SC was inoculated into 10 ml Eaton's broth (1:10 ratio) and grown in log phase at 37 ± 1 °C (5% CO_2) for 48 ± 72 hours. The same procedure was applied for 100 ml broth (1:10 ratio) and then subcultured into 1000 ml Thiaucourt broth (1:10 ratio). The grown culture (1 litre) was then centrifuged at 12,000 g at 4 °C. After discard the supernatant, pelet was washed 3 times with 100 ml sterile PBS at 12000 g for 40 minutes at 4 ° C. Resulted pelet was resuspended in 5 ml sterile PBS and then vortexed to homogenize the *Mycoplasma* suspension. Protein concentration of the *Mycoplasma* suspension was calculated by using the Pierce BCA Protein Analysis Kit (Thermo Scientific). Suspension was stored at -20 ° C until used (13).

SDS-PAGE and Western Blotting

After calculating the protein content of thawed *Mycoplasma* cells, sample proteins were electrophorosed on 4% stacking and 12 % resolving polyacrylamide gels at 40mA constant current. Electrophoresis is performed in the presence of the ionic detergent Sodium Dodecyl Sulfate (SDS) which gives proteins negative charges and under reducing and denaturing conditions. Proteins are separated by size (high molecular weight proteins on the top of the gel and low molecular weight proteins on the bottom) and molecular weight of sample proteins was determined by using appropriate protein marker standards with apparent molecular weights. For SDS-PAGE analysis, each well was loaded approximately 50 µg protein and after electrophoresis gels were stained with Coomassie blue G-250(Thermo Fischer Scientific)

For IBT, proteins were transferred electrophoretically on 0.2µm nitrocellulose membranes (NC) at 70 V constant voltage for 1.5 hours (14). NC was blocked with PBS containing 5% BSA and %0.02 sodium azide for 2 hours, air dried and cut into strips 0.3 cm wide and stored -20 °C until used. For immunoblots, antigen strips were incubated with 1/10 diluted test sera for 1 hour and alkaline phosphatase conjugated anti bovine Ig G (1:30.000) was used as secondary antibody. After washing steps, blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) in alkaline phosphatase buffer (100 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, pH 9.5).

After drying the strips, they were examined for the presence of core IgG immunoblot profile of five specific

antigenic bands of 110, 98, 95, 62/60 and 48 kDa. Sera giving a similar immunological profile were considered as positive.

Statistical Analysis

The specificity of CFT and c-ELISA was calculated by using 2X2 table (1).

RESULTS

A total of 253 serum samples were collected from cattle which had respiratory findings. All sera were tested by CFT, c-ELISA and IBT. All sera examined were negative by IBT but 7 (2.8 %) and 2 (0.8 %) were positive by c-ELISA and CFT, respectively (Table 1).

Table 1. Comparison of CFT, IBT and cELISA results of the serum samples from cattle for the serologic diagnosis of CBPP

Total serum number	Serologic tests															
	CFT						c-ELISA						IBT			
	pos	%	Dbt.	%	Neg.	%	pos	%	Dbt.	%	Neg.	%	pos	%	Neg	%
253	2	0.8	2	0.8	249	98.4	7	2.8	25	9.9	221	87.3	0		253	100

The core Ig G immunoblot profile of five specific antigen bands with apparent molecular weights of 110, 98, 95, 62/60 and 48 kDa were not detected in any of the serum samples in IBT. Immunoblotting of some serum samples resulted in either 1 to 3 major bands or very faint bands in variable size (Figure 1). Most of the serum samples did not reacted with any antigen by showing no bands at all in the blot.

The only serum sample giving positive reaction in both CFT and c-ELISA reacted with 29, 35 and 60 kDa antigens. IBT was regarded as gold standard in order to calculate specificity of CFT and c-ELISA and it was determined as 99% and 97%, respectively.

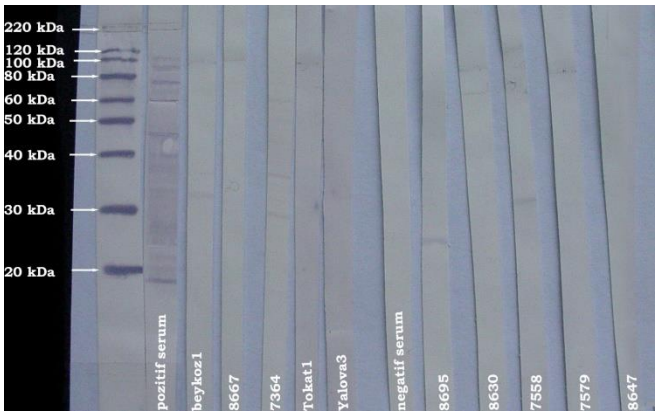

Figure 1. Western blot analysis of sera showed positive and doubtfull reactions by CFT and c-ELISA. Molecular markers are indicated on left in kDa

In SDS-PAGE analysis, proteins bands of 220, 78, 82, 68, 24 kDa belonging to various *Mycoplasma* species were found as common. The most different electrophoretic profile was seen in proteins of *M. capricolum subsp. capricolum* followed by *M. bovigenitalium* and *M.alkalescens* (Figure 2).

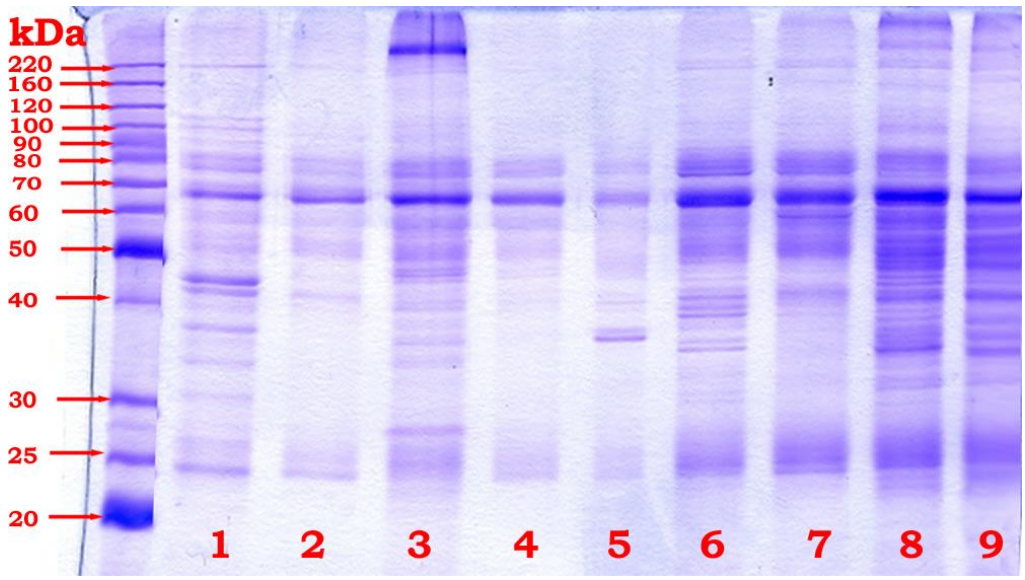

Figure 2: SDS-PAGE (12%) analysis of whole cell proteins of various *Mycoplasma* species. Molecular standarts are indicated in left as kDa. 1 *M. Bovigenitalium* 2. *M. Bovirhinis* 3. *M. capricolum subsp. capricolum* 4. *M.canadense* 5. *M.alkalescens* 6. *M. Bovis* 7. *Mycoplasma mycoides subsp. mycoides* SC V5 8. *Mycoplasma mycoides subsp.mycoides* SC PG1 9. *Mycoplasma mycoides subsp.mycoides* SC Botswana.

DISCUSSION AND CONCLUSION

For a proper diagnosis of contagious bovine pleuropneumonia, minimal cross reactivity among mycoplasmas is utmost important. However, various workers have common view that serological tests used in the diagnosis of the disease have relatively low sensitivity and specificity since there is no single serological test that determine every stage of CBPP and cross reactivity (2,6,5,7,10,15,16). Therefore, to develop more sensitive tests suitable for screening and afterwards to use highly specific and sensitive tests like IBT for confirming could be necessary for correct and appropriate serological diagnosis of the disease (5,6,7).

IBT allows the analysis of humoral immune response against many antigens of MmmSC in relate to electrophoretic profile of antigens. Therefore, non specific binding that might be observed in other serological reactions can largely

be eliminated. Moreover, humoral immune response to antigenic components of MmmSC can be detected after many months later. For this reason ,IBT seems to be important serological test in especially chronic and persistent cases.(2,5,6,7,19).

In addition to problems with sensitivity, the specificity of current official mass screening tests like CFT and c-ELISA is reduced due to cross reactions with other closely related (biochemical, antigenic, genetic) members of the *Mycoplasma mycoides* cluster, which can lead to false-positive results. In order to demonstrate if mycoplasma species reveal similar electroprofile with each other several *Mycoplasma* strains were used for this comparison, namely MmmSC Botswana (African strain), Mmm SC PG1 (referens strain, Mmm SC V5 (Australian strain), *M. Bovigenitalium*, *M. Bovirhinis*, *M. capricolum subsp. capricolum, M.canadense, M.alkalescens* and *M. bovis*. SDS-PAGE analysis of this strains demonstrated that antigens of 220, 78, 82, 68, 24 kDa were common in these strains. Cheng et.al. (20) reported that MmmSC shows cross reactions with *M bovine* group 7 and M.*capricolum subsp capricolum* in Mycoides cluster. This cross reaction was also observed in certain extent with *M.bovis* and *M. bovirhinis*.

According to the results, all positive and doubtful reactions by CFT and c-ELISA and all non specific bands observed in IBT were conidered as cross reactions with closely related mycoplasmas. Therefore, it was thought that all the suspected CFT and c-ELISA results need to be confirmed by IBT. Çetinkaya et. al.,(2003) performed bacteriological cultures from 62 suspected lung and they made PCR to identify MmmSC and suspected isolates. No MmmSC was isolated but they reported that *M. alkalescens, M. canadense, M. bovis* and *M. bovigenitalium* were isolated.

In an active surveillance performed in Sweetzerland for CBPP, 200.000 cattle slaughtered in slaughterhouse were included in the study. They found the lesion prevelance as 0.04%. The sera taken from suspected animals were tested by CFT and low titers (no more than 1/10 +++) were found as 1.6% . The researchers failed to isolate MmmSC from lesions but on the other hand they isolated *M.bovis* from 7 cases (17). In Hungary, 1248 serum samples were tested by CFT, and only 7 serum samples were found as positive with low titer (1/10 ++) and other serum samples were found as negative. No MmmSC isolate was made from 953 lung samples from slaughtered animals but *M.bovis* was isolated frequently (18). In our study we found low positivity rate (0,8%) by CFT and all positive ones had low titers. In this context, this positivity was evaluated as cross reaction with other genetically close species. This findings were compatible with other researcher's findings (17,18)

Specificity of CFT and c-ELISA were 99% and 97%, respectively. These findings were compatible with the researchers who found the specificity 99,5% (6)and 98% (1)

Competitive ELISA (c-ELISA) developed in Montpellier, CIRAD revealed similar sensitivity with CFT but specificity of c-ELISA was found higher than CFT (4,20) In this study positivity rate was found as 2.8 % by c-ELISA and suspected reactors were found as 9.9%. In our study false positivity in c-ELISA was found higher than CFT. This findings were compatible with those of Le Goff ve Thiaucourt (1998)

That we found no positive reactors by confirmatory IBT may give a clue that CBPP may not be present in the country, however, it is utmost important an active surveillance system for CBPP, especially the abottoir-based system based on the detection of typical lesions followed by microbial analysis of the sampled organs, be established in order to reveal the real disease status in the country.

CONFLICT OF INTEREST

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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