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DETECTION OF ANTIGENIC VARIATION AMONG MYCOPLASMA STRAINS ISOLATED FROM TISSUE CULTURES ACCOMPANIED WITH PREPARATION OF SPECIFIC ANTISERA TO P45 AND P49

BY

S. A. A. EL-SHATER*; S. I. EISSA** and NAHLA R. EL-KHATIB**

*Serum and Veterinary Vaccines Institute, Abbassia, Cairo.
**Animal Health Research Institute, Dokki, Giza.

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SUMMARY

Comparison of polypeptide patterns of five mycoplasma strains isolated from tissue cultures (*M. orale*, *M. arginini*, *M. salivarium*, *M. hyorhinis* and *A. laidlawii* using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis was carried out.

The SDS-PAGE protein profiles of mycoplasmas isolated from tissue cultures showed two common antigens at 45 and 49 kilodaltons (P45 and P49). The immunoreactivity in Western blots of the strains using *M. arginini* and *M. hyorhinis* anti-rabbit sera proved that P45 was found to be more immunogenic than p49.

Triton X-114 phase partitioning has revealed the hydrophilic nature of P45 and P49. Antisera raised to electroeluted P45 and P49 reacted specifically with these proteins when assessed on immunoblots of the examined five mycoplasma strains.

INTRODUCTION

Over the past three decades mycoplasmal infection, which is capable of drastically altering the structure and function of host cells, has been a common occurrence in cell cultures. Mycoplasma and Acholeplasma of the order Mycoplasmatales are among the smallest living prokaryotes (0.2-2 Um in diameter) and lack a cell wall. Many species of mycoplasma exert no cytopathogenic effects on host cells making infection difficult to detect. In cell culture, the concentration of mycoplasma can easily reach 10^7-10^8 colony forming unit per millilitre without over turbidity (Hay et al., 1989). Mycoplasma species and strains have been differentiated by comparison of polypeptide profiles using polyacrylamide gel electrophoresis (Daniels and Meddins, 1973); Andersen et al., 1987).

The aim of the present work was to detect the common antigens among mycoplasmas contaminating cell cultures and to prepare specific antisera from these antigens.

MATERIAL AND METHODS

Mycoplasma strains and growth conditions:

Stock culture of *M. orale, M. salivarium, M. arginini, M. hyorhinis* and *A. laidlawii* were previously isolated from tissue cultures. Minimum-passage culture stocks were expanded by 10% (vol.: vol.) inoculation of a 48-hour seed culture into medium that contained, per liter, 21 gm PPLO broth base without crystal violet (Difco Laboratories, Detroit, Michigan), 100 ml yeast extract (Difco), 150 ml normal horse serum, one gram each of glucose and arginine-HCl, 0.02 gm phenol red, 100,000 I. U. penicillin G and 0.5 gm thallium acetate, After 72 hours incubation at 37°C{based on change of colour in the pH indicator (Thomas and Sharp. 1990)} the mycoplasma cells were collected by centrifugation (17,000 xg for 30 minutes) and were washed three times in phosphate buffered saline (PBS). The protein concentration of each

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washed cell suspension was determined by the method of Bradford(1976) and adjusted to 1 mg of mycoplasma protein/ml in PBS.

Reference antisera:

Specific antisera against *M. arginini, M. hyorhinis, M. orale, M. salivarium and A. laidlawii* were kindly obtained from Dr. S. Geary, Department of Pathobiology, University of Connecticut, USA.

SDS-PAGE Molecular Weight Standards (Low Range): were obtained from Bio-Rad Laboratories, California, USA.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Proteins of the various mycoplasma strains were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). A. 4% (wt: vol) stacking gel (1.5 mm thick) was used with a 7.5% (wt: vol) resolving gel.

Whole cells of each strain (approximately 100 Ug protein) and molecular mass standards diluted to the same volume were added to an equal volume of buffer sample (0.1 M tris-HCl, pH 6.8; 2.5% Sodium Dodecyl Sulphate; 10 % B -mercaptoethanol; 20% glycerol and 0.02% bromophenol blue) and boiled for 5 minutes. Samples and standards were then loaded into wells and electrophoresed under constant current (20 mA per gel through the stacking gel and 30 mA per gel in the resolving gel.

The proteins were stained overnight with 0.25% (wt:: vol) Coomassie brilliant blue R-250 and destained in 15% methanol and 5% glacial acetic acid with two to three changes.

Western blots: Electrophoretic transfer to nitrocellulose (NTC) paper was accomplished by the method of Towbin et a l., (1979). Electrotransfer was conducted for 2 hours at 50 v (6.6 v/cm) with 0.2 A at room temperature accompanied with stirring buffer with magnetic stirrer during electrophoresis. After transfer to nitrocellulose (NTC) the membranes were stained with Ponceau s (Sigma) and the molecular weight standards were marked. The NTC blots were blocked with 5% bovine serum albumin (BSA), 20% fetal bovine serum in tris-buffered saline (10 mM Tris, pH 7.3, 0.9% Na cl) for one hour at 37°C with gentle rocking. Both primary and secondary antibodies were diluted appropriately in TBST (10 mM Tris pH 7.2, 150 mM Na Cl, 0.5% Tween 20). Secondary antibodies were horseradish peroxidase (Zymed Laboratories, San Francisco, California) diluted 1: 1000. The blots were developed for 5-10 minutes using $H_2 O_2$ and 4- chloro-1-naphthol (Bio-Rad Laboratories, Richmond, California) as described by the manufacturer.

Triton X-114 phase partitioning:

A. laidlawii was pelleted at 10000 xg and resuspended in a buffer containing 10mM Tris (pH 7.2). 150 mM Na Cl,1 mM PMSF, and Triton X-114 (Sigma) at 4°C. Solubilization of *A. laidlawii* proteins was accomplished with gentle rocking at 4°C for 30 min. Insoluble materials were then removed by centrifugation at 25000 xg for 30 min at 4°C. The supernatant was then incubated 45 min at 37°C to allow condensation of the detergent phase which was then separated by centrifugation at 10000 xg for 5 min at 22°C. The upper, aqueous phase, was transferred to a new tube and chilled to 4°C and Triton X-114 was added to a final concentration of 1%. The lower (detergent) phase was adjusted to its original volume with buffer without the addition of Triton X-114.Both vials were rocked at 4°C for 15 min and then transferred to an incubator (37°C) for 45 min. Both vials were then centrifuged at 10000 xg for 5 min at 22°C . This cycle was repeated twice to ensure complete partitioning.

Proteins were precipitated from the final detergentsoluble phase with the addition of 9 vols of methanol and incubated at -70°C for 18 h, followed by centrifugation at 12000 xg for 20 min at 4°C. Extracted proteins were run on a 10% polyacrylamide gel and then blotted according to Towbin et al., (1979).

Electroelution and antisera production:

A. laidlawii proteins (1.5 mg) were separated in preparative 10% polyacrylamide gels. The

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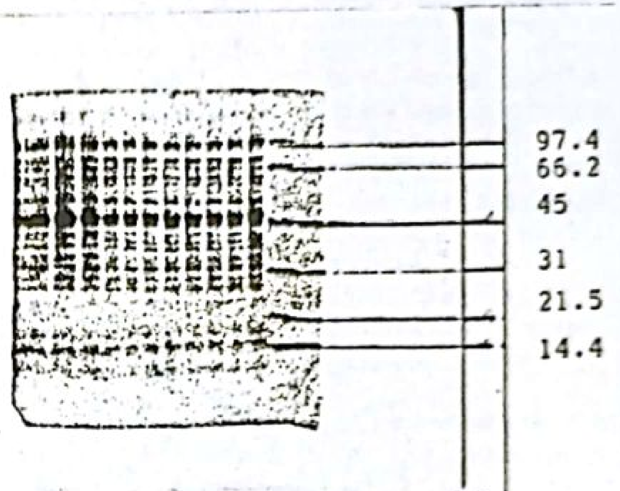
preparative gel was stained with coomassie brilliant blue R-250 (Sigma) and destained with 45% methanol, 10% acetic acid in distilled water. The coomassie-stained P45 and P49 bands were excised and electroeluted in a BioRad Electroelution Chamber in (192 mM Glycine, 15 mM Tris (pH 7.5), 0.1% SDS buffer) at 15 mA for 4h at 4°C. Electroeluted samples were run on a second preparative gel and electroeluted again to ensure the purity of P45 and P49. Specific antisera to P45 and P49 were produced by immunizing two adult, female, New Zealand White rabbits with 1mg of electroeluted protein with Freund's complete adjuvant. This was followed by two additional immunizations of lmg each with Freund's incomplete adjuvant at 10-days intervals. Blood was collected via the ear vein 7days after the final immunization.

RESULTS

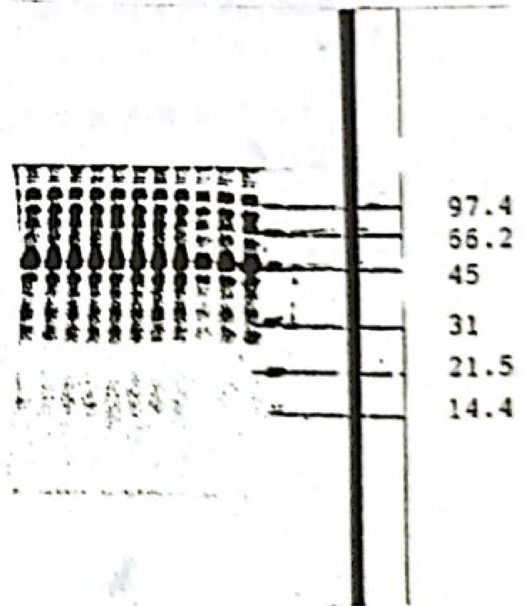
Commassie blue-stained SDS-PAGE profiles of *M. orale* and *A. laidlawii* (Figs. land 2) showed two common antigens at 45 and 49 KDa (P45 and P49). Immunoblotting of five mycoplasma strains was carried out to detect the common immunogenic proteins among the examined strains. Two proteins (P45 and P49) reacted with *M. arginini* and *M. hyorhinis* anti-rabbit sera diluted 1:200 (figs. 3 and 4).

The protein pattern of *A. laidlawii* whole cell protein as well as the aqueous and detergent phase were shown in (Fig. 5). The two common antigens (P45 and P49) could be detected in the aqueous phase.



A. laidlawii

Fig. 1: ***Acholeplasma laidlawii*** **proteins separated by SDS-PAGE**
Lanes 1-11: ***A. laidlawii*** **whole cell protein.**
Lane 12 : Molecular mass standards (Low Range).



M. orale

Fig. 2: SDS-PAGE protein profile of ***M. orale.***
Lanes 1-11: ***M. orale*** **whole cell protein.**
Lene 12 :Molecular mass standards (Low Range).

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Immunoblots of cell proteins of *M. arginini,** *M. orale,** *M. Salivarium,**** *M. hyorhinis***** and *A. laidlawii.**

Fig. 3: Reacted with *M. arginini* antiserum (diluted 1:200)

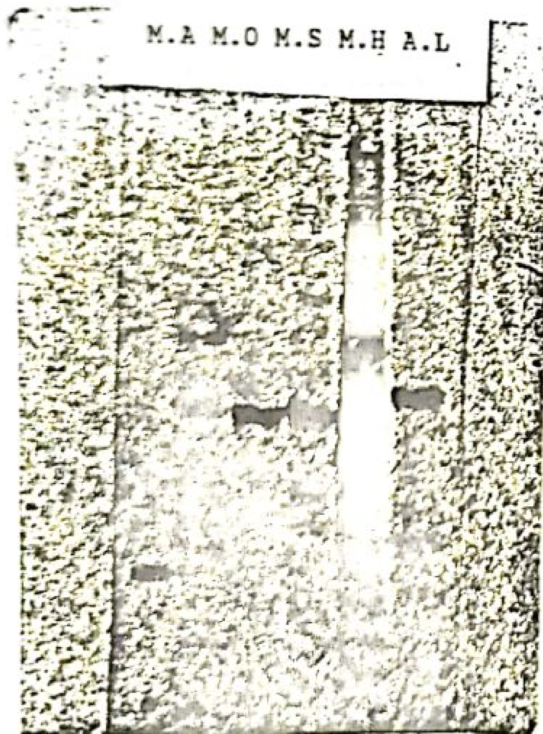
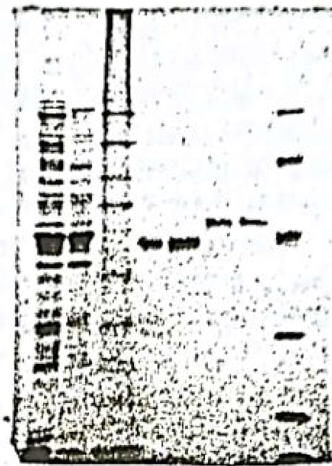


Fig. 4: Reacted with *M. hyorhinis* antiserum (diluted 1:200)



M. orale

Fig. 5: Coomassie blue-stained SDS-PAGE protein pattern of *A. Iaidlawii.*
From left Lane 1: *A. laidlawii* (whole cell protein).
Lane 2: *A. laidlawii* (aqueous phase).
Lane 3: *A. laidlawii* (detergent phase).
Lanes 4-5: P45
Lanes 6-7 P49
Lane 8 : Molecular mass standards (Low Range).

DISCUSSION

The present work is considered the first trial in Egypt for detection of common antigens and preparation of specific antisera to these antigens (P45 and P49) for rapid detection of mycoplasma contaminating cell cultures.

In this study, five mycoplasma strains previously isolated from cell cultures were examined for the detection of the immunogenic common antigens among the strains.

SDS-PAGE protein profiles of *M. orale* and *A. laidlawii* showed two common proteins at 45 and 49 kilodaltons (P45 and P49). Immunoreactivity in Western bolts of the strains using *M. arginini* and *M. hyorhinis* anti-rabbit sera proved that P45 was found to be more immunogenic than p49. Avakian et al., (1992) concluded that LP56 of *M. gallisepticum* responded relatively strong than LP64 to sera of turkeys experimentally infected with S6 strain.

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Immunoblots of the examined strains indicated that P45 and P49 were highly immunogenic in rabbits at four weeks post-infection. Forsyth et al., (1992) concluded that *M. gallisepticum* LP64 was highly immunogenic in chickens.

Hydrophobicity analysis of P45 and P49 was performed by Triton X-114 phase partitioning, revealed that the two proteins were found in the hydrophylic aqueous phase. *M. gallisepticum* LP64 partition was detected by Forsyth et al., (1992) in the hydrophobic detergent phase.

The use of specific antisera against P45 and P49 was recommended for the rapid detection of mycoplasma contaminating cell cultures.

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