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COMPARISON BETWEEN MYCOPLASMA GALLISEPTICUM F.STRAIN AND LOCAL FIELD ISOLATES BY USING SDS-PAGE AND WESTERN BLOT ANALYSIS

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Abstract

Various strains of *M.gallisepticum* (MG) including F-strain (as a reference) were examined by SDS-PAGE. Minor but distinct and reproduced differences in protein banding patterns were detected between strains. Cross reactivities among MG strains were examined by using rabbit polyclonal antisera. The major antigens and cross reacting epitopes of the MG strains were demonstrated by immunoblotting assay using homologous antisera.

INTRODUCTION

Mycoplasma infection is still one of the most important respiratory diseases of chicken which when complicated with other pathogens results in the so known as chronic respiratory disease which has a great economical importance in broilers, as well as laying birds (David and Avakian, 1992, Kleven *et al.*, 1992). Serological cross-reactivity between most of avian mycoplasma species complicates the diagnosis of avian mycoplasma (Abdel Moumen and Roy, 1995). The attenuated vaccine F strain of *Mycoplasma gallisepticum* is commonly used to immunize chickėn flocks against field exposure to wild-type MG (Kleven, 1981). Nothing is known about the interaction of vaccine strain with the wild-type MG under field conditions owing to the inability of currently serological tests such as : serum plate agglutination test, tube agglutination, haemagglutination inhibition, agar gel precipitation test and ELISA test (Hampson, 1985) to differentiate between the vaccinal and field MG strains. Bio-assay methods employed in-vivo and in-vitro have confirmed differences in pathogenic capacity among MG strains (Levisohn and Dykstra, 1987). Genotypic or phenotypic diversity among MG strains have been reported and polymorphism of proteins has been demonstrated among MG strains by sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Khan *et al.*, 1987, Zaho and Yamamoto, 1989). Immunogenic diversic associated with polymorphic proteins has been demonstrated by Western blot analysis (Thomas and Sharp, 1988).

The present study was undertaken to : 1) describe the protein patterns of *Mycoplasma gallisepticum* reference "F" strain (vaccinal strain) and MG local field isolate strains using SDS-PAGE test; 2) demonstrate immunogenic diversity associated with this polymorphic proteins by Western blot analysis using homologous antisera.

MATERIALS AND METHODS

1. Strains

a. *M.gallisepticum* reference "F" strain: It is the vaccinal strain obtained from Schering Plough, Animal Health.

b. *M.gallisepticum* local field isolate strains: Fifteen local field isolates of MG were isolated from diseased chicken. They were propagated and indentified as follows:

1. Isolation and purification of isolates through 45 m millipore filter (Stipkovits *et al.*, 1975).
2. Biochemical identification:

a. Digitonin sensitivity (Erno and Stipkovits, 1973).

b. Glucoe fermentation (Sabry, 1968).

c. Tetrazolium reduction test (Erno Stipkovits, 1973).

3. Serological identification Growth inhibition test (Clyde, 1964).

2. *Mycoplasma* Growth Media

All fifteen local field isolates and the reference "F" strain of *Mycoplasma gallisepticum* were propagated on liquid and solid media containing PPLO broth base (1.47%) and PPLO agar (2.47%) (Difco). The media base was enriched with 15% heat-inactivated fetal calf serum; 1% each glucose and arginine and 10% yeast extract.

The yeast extract was prepared as follows: 25.0 g of active yeast extract was sprinkled over the surface of 100 ml deionized water and the mixture was heated to boiling. The suspension was centrifuged at 2000 x g for 45 minutes at 20^{o}C and the clarified supernatant with pH adjusted to 8.0 was filtered, sterilized through a sequence of 0.8, 0.4 and 0.2 um-pore size filters. Nicotinamide Adenine Dinucleotide (NAD) was added to the growth media. Thallium acetate 1% and 0.5% penicillin-G sodium salt were added to prevent the contamination.

3. Antigen preparation

Antigen of each mycoplasma "F" strain and the fifteen local field isolates for immunoblot analysis were produced from stock cultures in broth medium.Each mycoplasma strain was subcultured three times in PPLO broth described above at a dilution of 1:10 (v/v). The final dilution (100 ml) of rapidaly growing culture of each strain was subcultured in 900 ml of broth. After incubation for 24 hours at 37^{o}C, the antigen was harvested by centrifugation at 40, 000 xg for 30 minutes at 4^{o}C (Douma *et al.*, 1989). The culture sediment was suspended in phosphate buffered saline (PBS) at pH 7.2 and washed three times by centrifugation. The final wash was diluted to a protein to a protein concentration of 1 mg/ml. The mycoplasma antigens were divided into 1 ml aliquots and stored at-20^{o}C.

4. Preparation of polyclonal rabbit antisera

The antigen for immunization was prepared as described above, except that the organisms were grown in broth medium containing 15% rabbit serum. The immunization protocol was essentially adopted as described by Martinez *et al.* (1990). Briefly, for each of fifteen and "F" strains of mycoplasma, two adult white New-Zealand rabbits were injected with the antigen emulsified in equal volumes of Freund's incomplete adjuvant and with antigen without adjuvant. The first series of injections consited of 1) 25 µg of antigen (total volume 1ml) given I/D on more the 10 sites along the back; 2) 25 µg of antigen (total volume : 1 ml) injected I/M into the thigh muscles at two sites and finally 3) 10 µg of antigen in 0.2 ml of PBS without adjuvant injected I/V in the ear veins. A second series of injections, using the same procedure, was initiated 3 weeks later using 15 µg of antigen for I/M route and 10 µg of antigen for I/V route, followed at 6, 7, 8 and 9 by injection of 15 µg of antigen without adjuvant given I/V. After one month rest, a final series of injections was made repeating the same schedule for the second series of injection.

Rabbits were bled 10-15 days after last inoculation. The blood was obtained and serum was collected and stored at - 20^{o}C.

5. Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-page)

The whole cell proteins of all strains of mycoplasma were separated by SDS-PAGE according to the discontinuous buffer system of Laemmli (1970) using 10% (w/v) acrylamide separating gel with 4% (w/v) stacking gel. Whole cells of each

strain (approximately 60 μg protein/well) mixed with an equal volume of loading buffer (0.5 M tris Hcl, pH 6.8), 10% (v/v) glycerol, 10% SDS (w/v) and 0.2% bromophenol blue), boiled for 5 minutes and then, loaded in separate wells onto 1.5 mm-thick gel. The molecular weight of protein bands was detesmined b running on the same gela prestained molecular weight standard (Bio-Rad). Electrophoresis was run under a constant current of 10 mA per gel for 45 minutes. All separated proteins were stained for 30 minutes with 0.1% Coomassie brilliant blue R-250 in fixative solution (40% methanol and 10% acetic acid) and destained with 30% methanol and 10% acetic acid for 2-3 hours with 2 to 3 changes. Finally, the gel was immersed in stop destain solution (5% methanol and 5% acetic acid) overnight at 20°C.

6. Western blot

The proteins of each of mycoplasma strain were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose (NC) membranes (Bio-Rad) according to the method of Towbin *et al.* (1979). Cross reacting antigens of *M.gallisepticum* strains were evaluated by immunoblotting. Transfer was performed in an electroblotting unit with a constant current of 30 mA applied to the gel-NC sheet Sandwich for 4 hours in transfer buffer (25mM tris-HCl, 192 mM glycine and 20% methanol (v/v) pH 8.3). After transfer, NC membranes were washed 3 times for 5 minutes each with PBS and blocked with PBS containing 0.25% gelatin for 2 hours. NC membranes were then washed 3 times for 5 minutes each in PBS containing 0.5% tween 20 (PBS-Tween). Washed NC sheets were cut into strips which were placed into an incubation tray (Bio-Rad). Then, the prepared rabbit polyclonal antisera were added into a groove in the incubation tray leave overnight at 4°C, using plateform tray for shaking. After 3 washings with PBS-tween, strips that reacted with polyclonal antisera were treated with peroxidase conjugated goats antirabbit IgG, and incubated for 1 hour at 20°C with gentle shaking. After 3 additional washings, bounded antibodies were visualized by developing solution containing 4-chloro-1-napthol (Bio-Rad) and hydrogen peroxide. Colour development was stopped by several washings in distilled water.

RESULTS

As shown in Table 1, 95 amples taken from 60 living ailing and 35 diseased dead broiler chicken (20 tracheal swabs, 18 palatine cleft swabs, 16 nasal cleft swabs, 6 nasal sinus exudate, 11 trachea, 15 air sacs, and 9 lung tissues) were examined for mycoplasma. Recovery rate of *M.gallisepticum* was 20%, 11.1%, 12.5%

and 33.3% from tracheal swabs, palatine cleft swabs, nasal swabs and nasal sinus exudate, respectively. Regarding the diseased dead chicken, it was 18.2%, 13.3%, 11.1% from trachea, air sacs and lung, respectively.

The results of SDS-PAGE in this study as shown in Figs. 1 and 2 revealed that minor easily identified differences in protein patterns existed among the reference strain (F strain) (Fig. 3) and 15 field isolated strains used in this study. It was revealed that, there were common electrophoretic patterns of cell protein, especially, among the molecular weight masses of 116 kilodalton (KDa) and 65KDa level constituting a high percent of total protein matter. According to the minor difference in protein patterns of *M.gallisepticum* field isolates used in this study could be differentiated into 6 subspecies as shown in Figs. 4,5,6,7,8,9. The reactivity patterns of the antisera with protein antigens of MG showed strong reacting bands with MG F strain and field isolates, especially, that possessed molecular weights of 116 KDa and 65 KDa, and also at 97 KDa with some of the field isolates (as shown in Fig. 10). According to the results obtained by analysis of immunoblots of MG strains, it was clear that the major reactions between them concerned the major polypeptide antigen. All of the results obtained were interpretated and compared with a negative control.

DISCUSSION

Trials for the isolation and identtification of *M.gallisepticum* from the respiratory organs of the affected chickens showed that the highest recovery rate was obtained from the nasal sinus exudate of living ailing chickens, (33.3%) followed by the tracheal swabs of living chickens (20%) and trachea of diseased dead chickens (18.2%). Meanwhile, nasal cleft swabs of living ailing chickens gave a recovery rate of 12.5%, palatine cleft swabs (11.1%) and air sacs (13.3%). On contrast, the lowest rate was obtained from the lung tissue samples (11%) and the total recovery rate of *M.gallisepticum* from broiler chickens was 15.8 as shown in Table 1. Similar results were also obtained by Edward and Kanarek (1960) who found that *M.gallisepticum* was common in the upper respiratory tract of fowls. Also, Koshimizu *et al.* (1978) isolated mycoplasms from 34 out of 164 throat swabs of chickens. Moreover, Branton *et al.* (1984) indicated that, swabs taken from the palatine cleft region yielded higher isolation rates and were more easily obtained than from tracheal swabs. Also, Ulgen and Kahraman (1993) identified *M.gallisepticum* from broiler and layer flocks.

In the present study, both SDS-PAGE and Western blot analysis proved capable of assessing relationship among MG strains. Interpretations of the results from both

techniques were in general agreement with those of David and Avakian (1992). The SDS-PAGE profile demonstrated that the field isolates were found to have two common protein patterns at the molecular weight of 116 KDa and 65 KDa. Similarly, EL-Shater *et al.* (1993) reported that the SDS-PAGE of local isolates of MG revealed that they have two common protein patterns. The findings of unique protein patterns of the vaccinal F-strain from various sources may be useful for their differentiations from field strains of MG (Khan *et al.*, 1987). TThe immunoblotting, using polyclonal polyclonal antisera for mycoplasma species helped to identify the major antigens and the nature of cross reactivity among the field isolates of MG.

The immunoreactivity in immunoblot analysis indicated diversity among the MG strains, whereas, the reactivity revealed the presence of several epitopes common to most of strains as reported previously by Bradley *et al.* (1988). Also, Avakian and Kleven (1990) identified the immunogenic species specific proteins of MG with relative molecular masses of 82, 65 and 35 KDa. In this particular case, the antigens of molecular weights of 116 and 65 KDa appeared to display major cross reaction between MG strains which confirm the genetic and antigenic relationship for these strains (Yogev *et al.*, 1989).

Table 1. Biochemical and serological identification of *M.gallisepticum* field isolates.

Type of examined sample / Test		Total no. of examined sample	Glucose fermentation	Digitonin sensitivity	Tetrazolium reduction	Growth inhibition test	No. of positive isolates	Recovery rate (%)
Living ailing Chicken	Tracheal swabs	20	17	12	7	4	4	20
	Palatine Cleft swab	18	11	9	5	2	2	11.1
	Nasal Swabs	16	6	4	3	2	2	12.5
	Nasal sinus exudate	6	5	3	2	2	2	33.3
	Total	60	39	28	17	10	10	16.7
Diseased dead chicken	Trachea	11	9	7	4	2	2	18.2
	Air sacs	15	11	9	3	2	2	13.3
	Lungs	9	6	3	1	1	1	11
	Total	35	26	19	8	5	5	14.3
Total of examined chicken		95	65	47	25	15	15	15.8

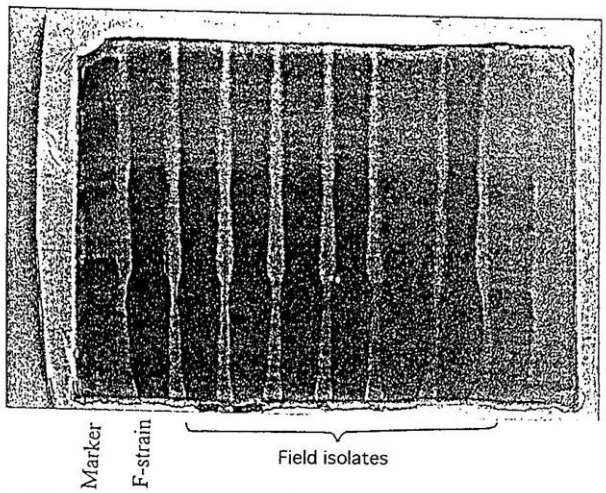


Fig. 1. SDS-PAGE protein patterns of *M.gallisepticum* (field isolates) isolated from infected chicken compared with reference "f" strain.

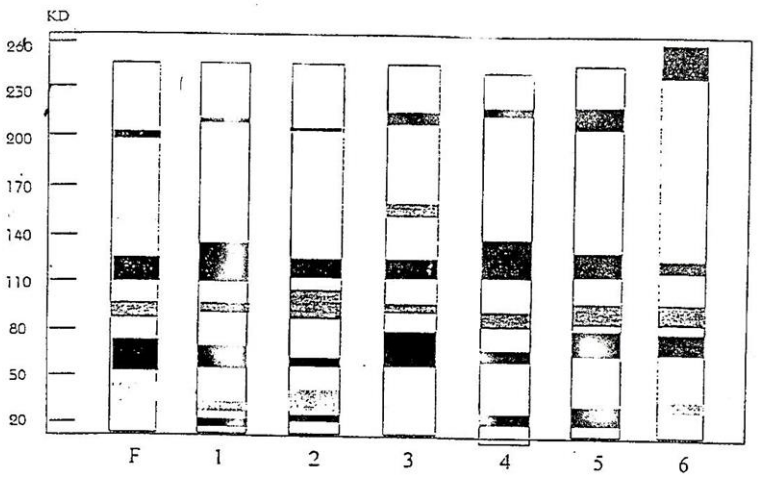


Fig. 2. Comparison of SDS-PAGE protein patterns of *M.gallisepticum* F strain and different field isolate groups.

PK	Mw	% Amt
1	200	5.54
2	116	19.78
3	97	11.64
4	65	39.36
5	42	23.68

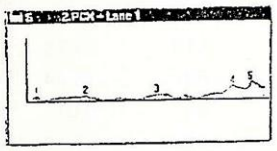


Fig. 3. Scanning of SDS-PPAGE protein patterns of *M.gallisepticum* F-strain (Reference strain).

* PK : Peak
* Mw : Molecular weight (KD).
* % Amt: % of each protein fraction to all.

Fig. 4. Scanning of SDS-PPAGE protein patterns of *M.gallisepticum* Field isolates. (6 serovars).

PK	Mw	%Amt
1	215	2.99
2	116	40.61
3	97	8.39
4	65	17.21
5	33	15.29
6	21	15.51

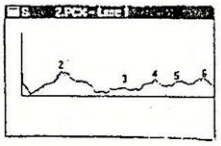


Fig. 5.

PK	MW	% Amt
1	206	1.26
2	116	23.75
3	94	35.04
4	65	8.01
5	43	26.50
6	31	5.44

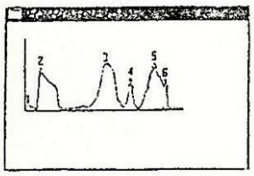


Fig. 6.

PK	MW	%Amt
1	210	9.50
2	157	9.98
3	116	22.06
4	97	8.62
5	65	49.84

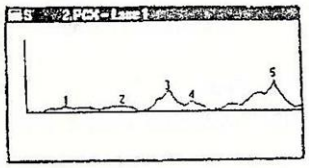


Fig. 7.

PK	MW	%Amt
1	216	5.67
2	116	48.85
3	81	13.23
4	65	9.47
5	31	22.78

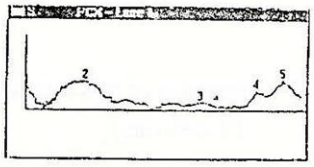


Fig. 8.

PK	MW	%Amt
1	205	17.34
2	116	19.86
3	80	16.79
4	65	29.68
5	31	16.33

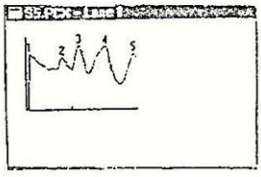
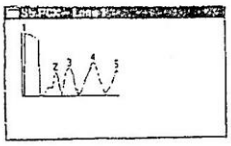


Fig. 9.

PK	MW	% Amt
1	246	42.88
2	116	9.65
3	97	14.11
4	65	23.42
5	45	9.94
*Pk	Peak.	



* Mw : Molecular weight (KD).

* % Amt : % of each protein fraction to all.

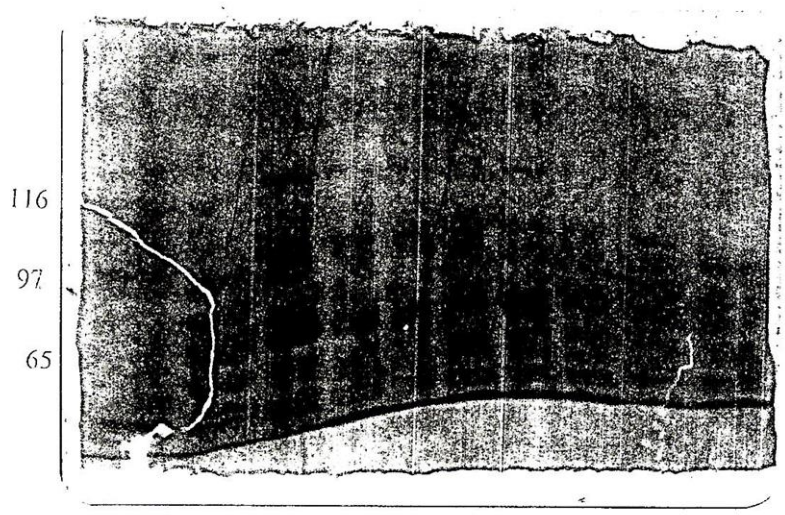


Fig. 10. Western blot analysis of *M.gallisepticum* (MG) reference "F" strain and local field isolates reacted with polyclonal antisera.

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تصنيف العترات المختلفة للميكوبلازما جاليسبتكم باستخدام تحليل البولى اكريلاميد جيل اليكتروفوريسيس والوسترن بلوت

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المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية-مركز البحوث الزراعية - جيزة - مصر.

تم فى هذه الدراسة فحص عترات مختلفة لميكروب الميكوبلازما جاليسيبتكم (المعزولة محلياً) ومقارنتها بعترة (ف) لنفس الميكروب (عترة مرجعية) وذلك باستخدام تحليل البولى اكريلاميد جيل اليكتروفوريسيس. ومن الدراسة اتضح انه يوجد فروق بسيطة ولكنها واضحة فى تتابع نموذج الشريط البروتينى بين مختلف العترات. وباستخدام تحليل الوسترن بلوت وجدت تفاعلات تداخلية بين عترات الميكوبلازما جاليسيبتكم المختبرة وذلك باستخدام مصل متعدد النوع المحضر فى الأرانب. وباستخدام المصل المضاد المتجانس فى تحليل الوسترن بلوت تم تعيين الانتيجيات الأساسية وكذلك الابيتوب المسئول عن التفاعل التداخلى لعترات الميكوبلازما جاليسيبتكم. ومن هذه الدراسة يتضح دقة وسرعة التصنيف باستخدام الأساليب الحديثة مقارنة بالطرق التقليدية.