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PREPARATION OF *MYCOPLASMA GALLISEPTICUM* SUBUNIT VACCINE AND ITS EVALUATION IN CHICKENS

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SUMMARY

A 64 kilodalton (KD) lipoprotein was isolated from Mycoplasma gallisepticum membrane and used for preparation of subunit vaccine. Evaluation of the prepared vaccine against colonization of *M. gallisepticum* (MG) in chicken trachea was studied. Immunoblot of 64 KD protein of *M. gallisepticum* separated by high performance liquid chromatography (HPLC) and Triton X -114 extraction, against anti- MG chicken serum proved that it is highly immunogenic protein. Sixty one-day old mycoplasma free Hubbard chicks were used. The chicks were divided into 5 groups each of tweleve chicks. Three groups were given 0.25, 0.5 and 0.75 mg of MG vaccine then challenged one month later with 15000 CFU/ 0.1ml /bird/ intratracheally. The remaining two groups were kept as negative and positive controls. Chickens vaccinated with subunit vaccine (0.5 and 0.75 mg/ bird) resulted in 100% protection against tracheal *M. gallisepticum* colonization . Birds of the group vaccinated with 0.25 mg / bird (group No.1)were positive for mycoplasma isolation up to 4 weeks post-challenge. Vaccinated groups No (2 and 3) were positive by serum plate agglutination test and ELISA along the duration of experiment. While chickens of group No (1) that vaccinated with 0.25 mg / bird gave suspected results by SPA and ELISA in the first week post-challenge, then gave positive results.

Vaccination of the chickens with *M. gallisepticum* subunit vaccine resulted in high antibody response to 64 KD protein at 2 weeks after the booster dose.

INTRODUCTION

Inactivated oil emulsion bacterin and live F-strain vaccines are currently commercially available to control the problem of *Mycoplasma gallisepticum* (MG) infection in chickens. The use of these vaccines resulted in incomplete protection rates against a large challenge dose (Yoder, 1979; Glisson and Kleven, 1984 and Kleven et al., 1984). Using a low challenge dose of 1500 colony-forming units of *M. gallisepticum* per layer, vaccinated with two dose of oil-emulsion MG bacterin resulted in only 40% protection rate against tracheal MG colonization (Talkington and Kleven, 1985).

The adhesin of MG has been reported and characterized by Kahane et al. (1984). It could be isolated by electroelution technique (Barbour et al, 1989). Vaccination with the subunit (MG-adhesin) bacterin resulted in antibody response specific to adhesin band (75KD) at 3 weeks post-inoculation of the first and second vaccination (Barbour and Newman, 1989).

The aim of the present study was the preparation of subunit vaccine used for protection of chickens against colonization of the *M. gallisepticum* in their respiratory tracts.

MATERIAL AND METHODS

Culture passage:

M. gallisepticum (PG-31 strain) was grown in Frey's medium (Frey et al., 1968) for 48 hours and harvested by centrifugation at 14000 r.p.m. for 20 minutes. Pellet was washed for three times with phosphate buffer saline (PBS) at pH7.2. The pellet was resuspended in PBS and the protein concentration was estimated as described by Bradford (1976).

Extraction of integral membrane proteins of *Mycoplasma gallisepticum* (Forsyth et al., 1992):

M. gallisepticum pellet was subjected to Triton X-114 partitioning in buffer containing 10mM Tris (pH 7.2), 150 mM NaCl, 1 mM phenyl methylsulfonyl fluoride (PMSF, Sigma), and 1 % Triton X-114 at 4°C. Solubilization of *M. gallisepticum* proteins was accoplished with gentle rocking at 4°C. for 30 min. Insoluble materials were then removed by centrifugation at 25000xg for 30 minutes at 4°C. The supernatant was then incubated 45 minutes at 37°C to allow condensation of the detergent phase which was then separated by centrifugation at 10000xg for 5 minutes 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 centrifuged at 10000 x g for 5 minutes at 22°C. This cycle was repeated twice to ensure complete partitioning.

Proteins were precipiated from the final detergent - soluble phase with the addition of 9 volumes of methanol and incubation at -70°C for 18 hr., followed by centrifugation at 12000 x g for 20 minutes at 4°C. Extracted proteins were run on a 10 % polyacrylamide gel and western blotted.

SDS - PAGE technique:

Electrophoresis was performed as described by Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250 (Sigma), and destained with mixture of 45% methanol ,10% acetic acid and 45% distilled water .

Immunoblotting

Electrophoretic transfer to nitrocellulose (NTC) paper was accomplished as described by Towbin et al. (1979).

After transfer to 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 - buffer saline (10 mM Tris, pH 7.3, 0.9% NaCl), for 1 hr at 37°C with gentle rocking, The primary antibody was diluted (1:500) in TBST (10 mM Tris pH 7.2, 150 mM NaCl, 0.05% Tween 20). Secondary antibody (horse radish peroxidase) conjugate was diluted 1:1000 .The blots were developed for 5-10 minutes using H_2O_2 and 4-chloro-1-naphthol substrate.

Electroelution

The Coomassie - stained LP 64 band was excised and electroeluted in a Bio-Rad electroelution chamber in 192 mM glycine 15 mM Tris (pH 7.5), 0.1 % SDS at 50 mA for 4 hrs at 4 °C. Samples were run on SDS-PAGE gels and electroeluted again to ensure the purity of LP 64 KD. The protein concentration in the eluted LP 64 KD band was estimated, then used for preparation of MG subunit vaccine.

High Performance Liquid Chromatography (HPLC):

A Model P 580 and the detector Model UVD 170 S were used (Dionex, USA) ,the column employed for separation by size exclusion was GF-250XI column 21.2 mm ID X 250 mm (Zorbax).The mobile phase is 0.2 M disodium phosphate, pH 7. 0 . The flow rate 5 ml / min. and detection was by UV absorbtion at 210 nm.The fraction collector was Foxy, Jr (Isco, Inc., USA).

Evaluation of the prepared subunit vaccine against colonization of *M. gallisepticum* in trachea of chickens:

Sixty one-day old Hubbard chicks were proved to

be free from avian mycoplasmas by serum plate agglutination (SPA) test (Yoder, 1980), ELISA (Talkington et al., 1985) and by cultural method (Kleven, 1985). At 4 th week of age, three groups were vaccinated subcutaneously with three different doses of MG subunit vaccine (0.25mg, 0.5 mg and 0.75 mg per bird respectively). Second dose was given 2 weeks later (Table 1). At 8 th week of age, the birds were tested by SPA, ELISA and cultural methods to ensure that no avian mycoplasmas had infected the chickens. Challenge with low - passage *M. gallisepticum* (PG 31 strain) was grown in Frey's broth with 12 % swine serum and incubated at 37°C for 18 hours (15 000 CFU/0.1 ml for each bird) intratracheally.

Table (1): Experimental design for evaluation of the protection role of LP 64 band against colonization of *M. gallisepticum*..

	Groups				
	Vaccinated			Control	
	1	2	3	4 (-ve Control)	5 (+ve Control)
Dose of vaccination	0.25mg	0.50mg	0.75mg	-	-
No. of chickens	12	12	12	12	12
Route of vaccination	Subcutaneously				
Age of chickens at the first vaccination	4 weeks				
Age of chickens at the booster dose	6weeks				
Age of chickens at challenge	8 weeks				
Dose of challenge	0.1ml / bird (15000 CFU/bird)		-		0.1ml / bird (15000 CFU/bird)
Route of challenge	Intratracheal		-		Intratracheal
Collection of blood samples , trachea, air sac and lungs	After 1, 2, 4, 6 and 8 weeks (post-challenge)				

At 1, 2, 4, 6 and 8 weeks post-challenge, trachea, lungs, air sac and serum samples from all groups were tested for evaluation of the subunit vaccine against colonization of MG in the trachea of chickens.

Tweleve chicks (group No. 4) were used as negative control while another tweleve chickens (group No. 5) were considered as positive control infected and not vaccinated .

RESULTS

After extraction of *M. gallisepticum* with Triton X-114, eight hydrophobic protein bands (28, 38, 42, 48, 56, 64, 76 and 85 KD) were detected in the detergent phase (by SDS-PAGE electrophoresis) indicating that they were integral membrane proteins, five of them (28,38,56,64 and 76 KD)were immunogenic proteins (Figures 1 and 2). Following separation of *M. gallisepticum* proteins with high performance liquid chromatography (HPLC), three protein fractions were obtained, 64 KD protein of fractions 1 and 2 reacted strongly with anti-MG chicken serum, indicating its immunogenicity (Figs 3, 4).

Table (2) shows the re-isolation results of *M. gallisepticum* from the respiratory tract of different groups. Mycoplasma could not be re-isolated from groups vaccinated with 0.5 mg and 0.75 mg per bird as well as the negative control group (groups No. 2, 3 and 4). *M. gallisepticum* was isolated from all birds of the positive control group No. (5). Birds of group vaccinated with 0.25 mg / bird were positive for Mycoplasma isolation up to 4 weeks post-challenge.

Serum samples were collected from the different groups and tested by serum plate agglutination test (SPA) and ELISA. Chickens vaccinated with 0.50 mg and 0.75 mg / bird (groups No. 2 and 3) were positive for *M. gallisepticum* antibodies during all period of the experiment. While the group vaccinated with 0.25 mg / bird (group No. 1) was suspected to ELISA at the first week post - challenge, then was positive during the next weeks. The highest antibody titer was detected in chickens of group No.3 vaccinated with 0.75 mg / bird (1273, 2660, 1860, 1860, 2125) during weeks 1, 2, 4, 6 and 8, respectively. The least antibody titer after challenge was of group No.(1) which showed 362, 922, 982, 1179 and 1211 during weeks 1, 2, 4, 6 and 8, respectively (Table, 3).

The subunit vaccine given to chickens of groups No. 2 and 3 resulted in clear antibody response to LP 64 after 2 weeks of the booster vaccination (Fig. 5) Scans of immunoblots of *M. gallisepticum* indicated that LP 64 was highly immunogenic in vaccinated chickens. However, there was a cross reaction with the 76 KD protein and minor cross reactions to other bands were detected.

The antibody responses were prominent in sera of vaccinated chickens after challenge. The samples

Table (2): Re- isolation of *M. gallisepticum* from trachea, air sac, and lungs of chickens vaccinated with different doses of subunit vaccine after 1,2, 4, 6 and 8 weeks challenged intratracheally with MG (PG-31).

No. of Group	Week After Challenge	Vaccine dose/bird	Re-isolation of MG from					
			Trachea		Air Sacs		Lungs	
			Result	C.F.U.*	Result	C.F.U.*	Result	C.F.U.*
1	1st	0.25mg	Positive	10^{-}	Negative	-	Negative	-
2		0.50mg	Negative	-	Negative	-	Negative	-
3		0.75mg	Negative	-	Negative	-	Negative	-
4		-Ve Control	Negative	-	Negative	-	Negative	-
5		+Ve Control	Positive	10^{-}	Negative	-	Negative	-
1	2nd	0.25mg	Positive	10^{-4}	Negative	-	Negative	-
2		0.50mg	Negative	-	Negative	-	Negative	-
3		0.75mg	Negative	-	Negative	-	Negative	-
4		-Ve Control	Negative	-	Negative	-	Negative	-
5		+Ve Control	Positive	10^{-3}	Negative	-	Negative	-
1	4th	0.25mg	Negative	-	Negative	-	Negative	-
2		0.50mg	Negative	-	Negative	-	Negative	-
3		0.75mg	Negative	-	Negative	-	Negative	-
4		-Ve Control	Negative	-	Negative	-	Negative	-
5		+Ve Control	Positive	10^{-4}	positive	10^{-2}	positive	10^{-2}
1	6th	0.25mg	Negative	10^{-4}	Negative	-	Negative	-
2		0.50mg	Negative	-	Negative	-	Negative	-
3		0.75mg	Negative	-	Negative	-	Negative	-
4		-Ve Control	Negative	-	Negative	-	Negative	-
5		+Ve Control	Positive	10^{-5}	positive	10^{-3}	positive	10^{-3}
1	8th	0.25mg	Positive	-	Negative	-	Negative	-
2		0.50mg	Negative	-	Negative	-	Negative	-
3		0.75mg	Negative	-	Negative	-	Negative	-
4		-Ve Control	Negative	-	Negative	-	Negative	-
5		+Ve Control	Positive	10^{-5}	Positive	10^{-4}	Positive	10^{-4}

C.F.U*= Colony Forming Unit

Table (3): Results of serum plate agglutination (SPA)and ELISA titers from different groups vaccinated with 0.25 ,0.50 and 0.75 mg of subunit vaccine after 1, 2, 4, 6 and 8 weeks post-challenge with MG (PG-31).

No. of Group	Week After Challenge	Vaccine dose/bird	Serological Tests		
			SPA	ELISA	
				Titers	Interpretation*
1	1st	0.25mg	Weak positive	362	Suspicious
2		0.50mg	Positive	997	Positive
3		0.75mg	Positive	1273	Positive
4		-Ve Control	Negative	40	Negative
5		+Ve Control	Weak positive	154	Suspicious
1	2nd	0.25mg	Positive	922	Positive
2		0.50mg	Positive	1865	Positive
3		0.75mg	Positive	2660	Positive
4		-Ve Control	Negative	80	Negative
5		+Ve Control	Positive	738	Suspicious
1	4th	0.25mg	Positive	982	Positive
2		0.50mg	Positive	1290	Positive
3		0.75mg	Positive	1860	Positive
4		-Ve Control	Negative	62	Negative
5		+Ve Control	Positive	940	Positive
1	6th	0.25mg	Positive	1179	Positive
2		0.50mg	Positive	1446	Positive
3		0.75mg	Positive	1860	Positive
4		-Ve Control	Negative	60	Negative
5		+Ve Control	Positive	1430	Positive
1	8th	0.25mg	Positive	1211	Positive
2		0.50mg	Positive	1650	Positive
3		0.75mg	Positive	2125	Positive
4		-Ve Control	Negative	85	Negative
5		+Ve Control	Positive	1530	Positive

Interpretation*: Negative = 0 - less than 149 , Suspicious = 149 -744 and Positive =more than 744.

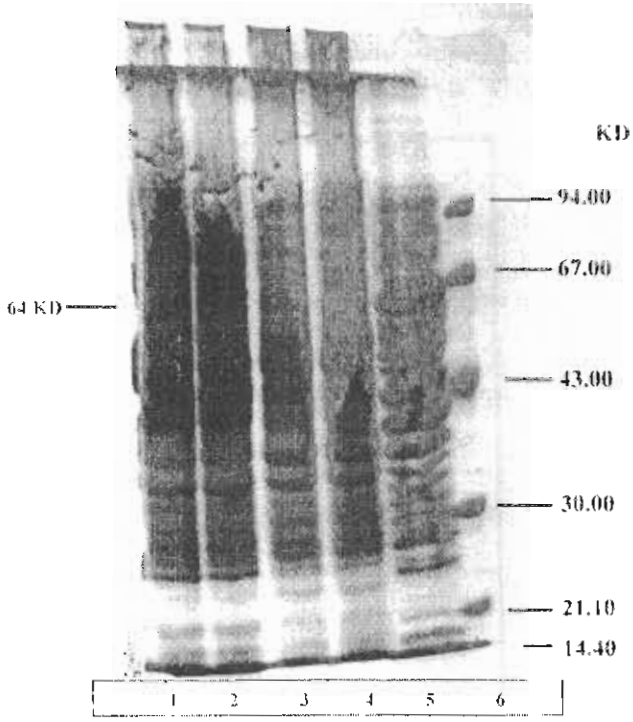

Fig. (1): SDS-PAGE protein profile of *M. gallisepticum* whole cell protein and detergent phase partitioned by Triton X-114.

Lanes 1, 2: *M. gallisepticum* detergent phase of non-sonicated antigen.

Lanes 3, 4: *M. gallisepticum* detergent phase of sonicated antigen.

Lane 5: *M. gallisepticum* whole cell antigen (pH 6.4).

Lane 6: Low molecular weight standard (Pharmacia).

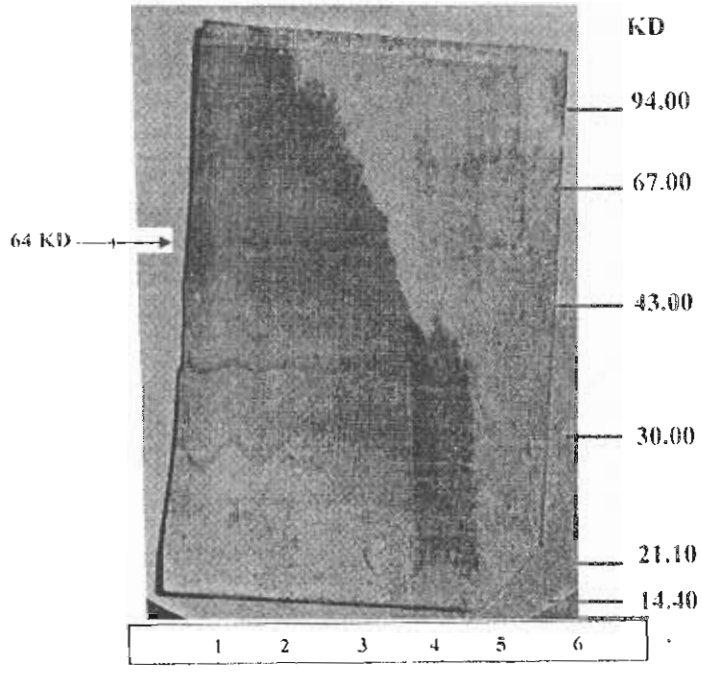

Fig. (2): Immunoblots of *M. gallisepticum* whole cell protein and detergent phase partitioned by Triton X-114 using anti-MG serum from chickens 30 days post infection.

Lanes 1, 2: *M. gallisepticum* detergent phase of non-sonicated antigen.

Lanes 3, 4: *M. gallisepticum* detergent phase of sonicated antigen.

Lane 5 : *M. gallisepticum* whole cell antigen (pH 6.4).

Lane 6 : Low molecular weight standard (Pharmacia).

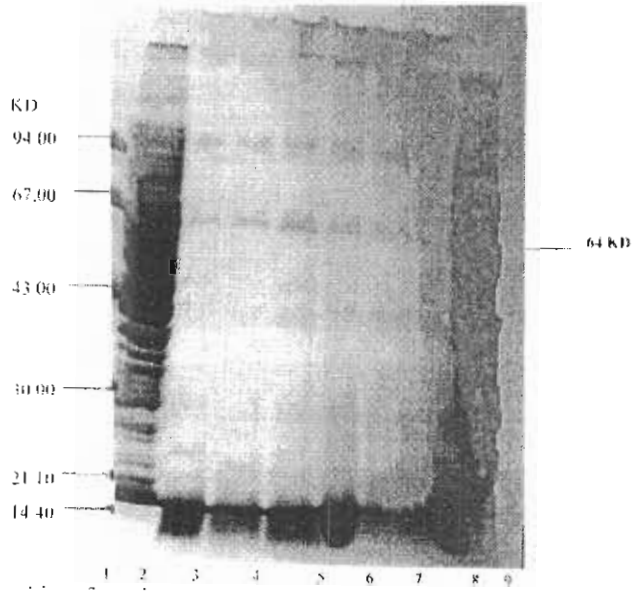

Fig. (3): Electrophoretic pattern of *M. gallisepticum* protein fractions separated by HPLC.

Lane 1: Low molecular weight standard (Pharmacia).

Lane 2: *M. gallisepticum* whole cell protein.

Lane 3: *M. gallisepticum* (Fraction 1, antigen pH 6.4).

Lane 4. *M. gallisepticum* (Fraction 2, antigen pH 6.4).

Lane 5: *M. gallisepticum* (Fraction 3, antigen pH 6.4).

Lane 6: *M. gallisepticum* (Fraction 1, antigen pH 6.8).

Lane 7: *M. gallisepticum* (Fraction 2, antigen pH 6.8).

Lane 8: *M. gallisepticum* (Fraction 3, antigen pH 6.8).

Lane 9: *M. gallisepticum* whole cell protein.

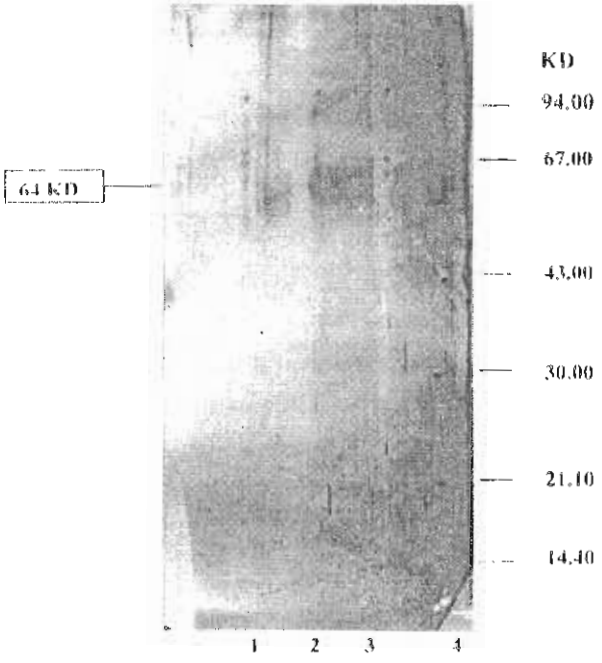

Fig. (4): Immunoblot of *M. gallisepticum* protein fractions separated by HPLC against anti-MG positive chicken serum.

Lane 1: *M. gallisepticum* (Fraction 3).

Lane 2: *M. gallisepticum* (Fraction 2).

Lane 3: *M. gallisepticum* (Fraction 1).

Lane 4: Low molecular weight standard (Pharmacia).

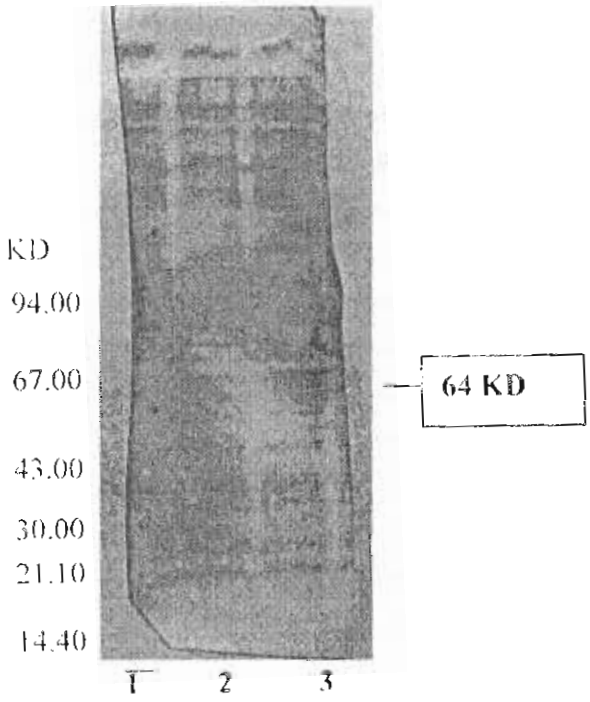

Fig. (5 : Immunoblot of *M. gallisepticum* against serum of chickens vaccinated with subunit vaccine (two weeks after second vaccination).
Lane 1: Low molecular weight standard (Pharmacia).
Lanes 2, 3: *M. gallisepticum* whole cell antigen.

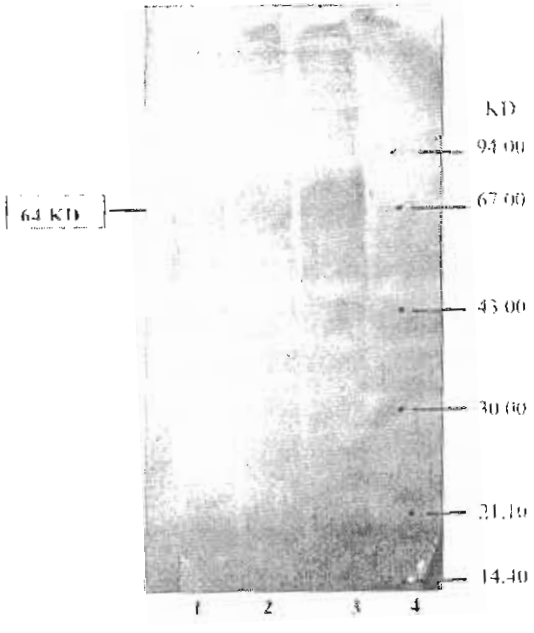

Fig. (6) : Immunoblot of *M. gallisepticum* against vaccinated chicken sera (two weeks post-challenge).
Lanes 1, 2, 3: *M. gallisepticum* whole cell antigen.
Lane 4: Low molecular weight standard (Pharmacia).

were collected one week up to 8 weeks post challenge. Clear antibody response to LP 64 was detected by immunoblotting against *M. gallisepticum*. However, a minor cross reaction of this antibody to the 48 KD protein was detected at the second week post challenge (Fig. 6).

DISCUSSION

In the present study, *M. gallisepticum* proteins were extracted with Triton X-114. About 8 hydrophobic proteins including LP 64 were detected in the detergent phase. Avakian et al. (1991) found that three immunogenic specific proteins (P64, P56 and P 26) of *M. gallisepticum* were integral membrane proteins, after extraction with Triton X-114. They concluded that these immunogenic proteins should be used in the development of a subunit vaccine for the prevention of M. gallisepticum disease in poultry. Forsyth et al (1992) found that at least seven hydrophobic proteins, including LP 64 appear to be lipid modified.This degree of hydrophobicity suggests that these proteins are integral membrane components.

In the present work ,three fractions were obtained by HPLC separation of *M.gallisepticum* proteins, 64 KD protein of fractions 1 and 2 reacted strongly with anti-MG chicken serum , indicating its immunogenicity. Kahane et al. (1984) isolated the adhesin protein 75 KD of *M.gallisepticum* by using column chromatography.

The protective effects of MG subunit vaccine were examined serologically and culturally. The subunit vaccine stimulated the humoral response in chickens as detected by SPA and ELISA. ELISA titers were the highest in group No. 3 (1273, 2660, 1860, 1860, 2125) during the weeks 1, 2, 4, 6, and 8 respectively (table 3). The least ELISA titres were in group No. 1 (362,922,982,1179 and 1211 during the same weeks respectively). These results are agree with that of Talkington and Kleven (1985) who studied the protective effects of oil -emulsion bacterin, which readily stimulated a strong humoral response in birds as detected by SPA and HI tests.

In the present work, the use of subunit vaccine for protection of chicken tracheas against colonization by the mycoplasma was studied. Mycoplasma could not be re-isolated from groups No. 2 and 3. However, group No.1 was positive for mycoplasma re-isolation up to 4 th week post-challenge (PC). Therefore, the subunit vaccine provided a pronounced protection of birds in groups No.2 and 3 against colonization by the mycoplasma (Table 2). Yagihashi et al. (1981) concluded that the use of bacterin resulted in good protection against infection by mycoplasma. Also, Kleven (1985) mentioned that the tracheal populations of *M. gallisepticum* were lower in the chickens up to 8 weeks post-challenge. Forsyth et al. (1992) found that anti-LP 64 serum inhibited attachment of radiolabelled *M. gallisepticum* to the trachea by more than 60%. This degree of in-

hibition suggests a role for LP 64 in the process of cytadherence.

The present study showed that the chickens vaccinated with subunit vaccine reacted strongly with 64 KD protein of *M. gallisepticum* in western blot, on the other hand , cross reaction with the 76 KD protein (pre-challenge) and with 48 KD protein (post-challenge).These results are in agreement with Barbour and Newman (1989) who found that the use of *M. gallisepticum* subunit vaccine prepared from the 75 KD adhesin for vaccination of chickens resulted in antibody response to the adhesin band at 3 weeks post the first and second vaccination , however,cross reactions to *M. gallisepticum* proteins of 85 KD and 56 KD were detected.

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