M9 Protein As An ELISA Antigen For Evaluation Of Immune Response To *Mycoplasma gallisepticum*

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ABSTRACT

PCR amplification of *M. gallisepticum* genome using the M9 gene primers succeeded to generate of DNA fragment of about 1 kb size. The results of PCR agreed with that obtained by biochemical and serological identification.

Regarding the molecular cloning of the PCR product using the specific primers of M9 gene, the PCR product ligated directly into the vector then transformed into the competent cells and the recombinant plasmids with the correct inserts were validated by many preparation and restriction followed by restriction digestion.

SDS-PAGE was an adequate method to identify the presence of 64kDa recombinant protein in the transformed cells. Also, Western blot analysis for such protein showed specific reactivity against specific *M. gallisepticum* antisera.

Using the recombinant protein as an ELISA antigen revealed diagnostic activity as early as the 6th day post infection. Such activity increased gradually till reached its maximal level (100%) with all infected chicken groups on the day 43 post infection.

INTRODUCTION

Concentration has been focused on one of the most pathogenic avian mycoplasmas which is *Mycoplasma gallisepticum* (MG), causing chronic respiratory disease (CRD) in either commercial broiler industry or egg layer flocks producing reduction of egg production, hatchability and down grading of carcasses. CRD is responsible for severe economic losses in poultry industry particularly when associated with respiratory pathogens *(1)*.

Mycoplasma cause subclinical upper respiratory disease that may become systemic and results in an acute or chronic infectious disease of chickens and turkeys. Also, horizontal transmission occurs readily by direct contact via the respiratory tract and vertical transmission from infected breeder hens to the progeny has played a major role in the initiation of outbreaks *(2)*.

Control of mycoplasmosis, particularly *M. gallisepticum* infection, is the key stone of modern intensive poultry industry. The economic impact is measured not only by the losses due to the disease, treatment and reduced productivity but also in expense of the diagnostic procedures needed to determine the mycoplasma status of breeding flocks *(3)*. Thus, considerable interest has been focused on the composition and properties of membrane antigens of several pathogenic mycoplasma species particularly MG *(4)*. Therefore, the need for highly sensitive and specific diagnostic antigen is evident.

Hence the objective of this study was to clone, express, characterize, standardize and evaluate a specific recombinant protein as an ELISA antigen.

MATERIL AND METHODS

Strains

Two references (S6 & F) *M. gallisepticum* strains and 23 clinical isolates were used in this study.

Extraction of Mycoplasma DNA.

Extraction of Mycoplasma DNA was carried out as previously described method *(5)*. A 100 ml of overnight culture growth from each mycoplasma strain was centrifuged at 13,000 rpm for 30 minutes. The cell pellets were

washed twice in 100 ul of 150 mM PBS (pH 7.2) and suspended in 25 µl PBS. The cell suspension was heated directly at 100°C for 10 minutes and cooled on ice for 5 minutes. Finally, the cell suspension was centrifuged at 15,000 rpm for 5 minutes, chromosomal DNA was collected and stored at 4°C.

Amplification of the desired gene using Polymerase Chain Reaction (PCR).

For amplification of the desired gene, PCR technique was performed as follows *(6)*. 1ul of DNA extracted in TE buffer was added to 49 ul of reaction mixed buffer that contained 10 mM tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 ul taq DNA polymerase and 100 ng of each primer (The first primer pair sequence for mycoplasma M9 gene;

Forward,5-GGTGGATCTGCTGGAATGCT-3,
Reverse,5-GCTGCAATCGCTTGGTGA-3,
and the primer pair sequence was;
Forward, 5-AGGCAGCAGTAGGCAAT-3,
Reverse, 5-CGTTCTCGGGTCTTGTA-3),

The selected primers were synthesized in Biosynthesis Corporation, USA. The DNA amplification was performed in a DNA thermal cycler under the following condition: 94°C for 3 minutes for one cycle (initial denaturation) followed by 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes for 35 cycles.

Agarose gel electrophoresis.

The amplified product was analyzed by an agarose gel electrophoresis *(7)*. The samples were electrophoresed at a constant 100 volts for 1 hour, visualized under ultraviolet light and photographed using gel documentation.

Cloning expression, detection and purification of recombinant protein.

Using Pin point Xa-1 T-vector system (Promega Corporation, USA) and Pin point Xa protein purification system.

(a) Ligation process

The ligation reaction was set up as follows.1 µl T4 DNA ligase 10x buffer, 1 µl plasmid Vector, 3 µl PCR product, 1 µl T4 DNA ligase, and 10 µl deionized water.

Then incubated at 15°C for at least 3 hours up to overnight. A 2ul aliquat of ligation reaction were transferred to a sterile microcentrifuge tube and placed on ice to be used for transformation.

(b)Transformation

Ligation product was centrifuged for 5 seconds and placed on ice. Frozen *E.coli* (strain JM109) competent cells (Promega, Maison, USA) was removed from -80°C storage and placed on ice until just thawed, gently mixed and then 50ul were transferred to ligation product tube then gently mixed and placed on ice for 30 minutes.

The cells then subjected to heat shock for 45-50 seconds in a water bath at exactly 42°C (without shaking). Then, the tubes returned to ice for 2 minutes and 950 ul of soc. medium was added. The tubes gently inverted to mix and incubated for 1 hour at 37°C with shaking. One hundred ul of transformation culture (and control reaction) was spread onto each LB/ ampicillin plates (and or LB/ampicillin/ IPTG/ XGal plates), then, incubated overnight at 37°C.

(c) Isolation of recombinant plasmid

1. Miniprep procedures

A 2 ml liquid culture carrying recombinant plasmid DNA were incubated overnight on shaker incubator at 37°C then 1.5 ml were centrifuged at 10,000 rpm for 2 minutes. Supernatant fluid was discarded and the cells were resuspended in 300 ul STET buffer then 25 ul lysozyme was added and mixed by inversion, and the tube was immediately placed in vigorously boiling water bath for 45 seconds.

The tube was centrifuged at 10,000 rpm for 5 minutes and the cell debris was removed. 350 ul isopropanol was added to the supernatant, mixed well and centrifuged at 12,000 rpm for 5 minutes. The pellet was resuspended in 25 ul TE.

2.Restriction digestion of recombinant plasmid DNA

20 ul of DNA plasmid were digested using SmaI and NruI (1 ul of each), 3 ul of digestion buffer and distilled water was added up to final volume of 30 ul. The reaction tubes were incubated at 37°C in water bath for 2 hours. The digested DNA was electrophoresed in 0.7% agarose gel 0.5x TBE buffer, stained with ethedium bromide (10 ul/ml) and the bands were visualized by examination under an UV transilluminator.

(d) Protein expression and detection

1. Expression of fusion protein

The host strain carrying the vector with the desired insert DNA was inoculated in LB broth containing biotin (2 uM final concentration) and ampicillin (100 ug/ml) with a freshly isolated bacterial colony, then incubated overnight at 37°C with shaking. The overnight culture was diluted 1:100 with fresh LB containing biotin and ampicillin and incubated an hour at 37°C with continous shaking. Protein expression was induced by adding IPTG (100 µM final concentration) to the culture and then incubated for 4-5 hours at 37°C with shaking.

2. Detection of protein

100ul broth culture were transferred to 1.5 ml micro-centrifuge tube, centrifuged at 13,000 xg for 5 minutes and the supernatant was discarded. 50 ul of sample 1x buffer was added and cells resuspended by vortexing then heated at 95°C for 5 minutes with associated vortexing (this treatment lysed cells and coat protein with SDS). 50 ul of the heat treated samples were loaded on a 10% SDS-PAGE.

(e) Purification of recombinant protein

The host strain culture with the vector and insert DNA were inoculated into LB broth containing biotin and ampicillin and incubated at 37°C with shaking then protein expression was induced by adding IPTG to the cultures and incubated for 4-5 hours at 37°C with shaking. Cells were harvested by centrifugation at 8000 xg for 10 minutes. Then, resuspended by stirring in 10 volumes (ml/gm cell past) of cell lysis buffer at 4°C or on ice. The suspension was sonicated by ten, 15 seconds pulses with a 5 seconds pause between pulses, for 2 minutes. The crude lysate was centrifuged at 1000 xg for 15 minutes at 4°C to remove cellular debris. The suspension was adjusted to a protein concentration of 3mg/ml.

before using the soft link resin for first time two resin bed volumes of a compatible stabilizing buffer (cell lysis buffer) were added containing 5 mM biotin and left for 15 minutes at room temperature. The soft link resin was regenerated by washing with 10% acetic acid and PBS.

Using a column capture, the resin in the column equilibrated using a stabilizing buffer compatible with the biotinylated molecule to be purified. The cell extract slowly applied (1ml/minute) to allow efficient capture then washed the column at least 5 column volumes of buffer. A stabilizing buffer containing 5 mM biotin was added to elute the protein then collecting fractions of 0.5 ml began immediately. Reinitiated the elution and continued to collect fractions then excess of biotin was removed by dialyzing against stabilizing buffer, then the protein ready for the subsequent assays.

Characterization of recombinant protein using Western blot analysis *(8)*.

Recombinant protein (30 ul/ml) was applied to 0.75 mm thick, 5% stacking/10% resolving SDS-PAGE gels and run overnight at 4 mA constant current/gel using running buffer. Then blotted onto nitrocellulose membrane pore size of 0.2µm at 30 V constant voltage for 2 hours, then the transferred protein was visualized by 0.1% amidoblack stain then destained. The nitrocellulose (NC) strips with transferred protein were blocked by incubation for 3 hours at room temperature in TBS containing 5% bovine serum albumin. The prepared antisera (prepared in Central Laboratory for Evaluation of Veterinary

Biologics) was diluted 1/80 in TBS containing 1% swine serum and 1% calf sera (TBS-sera). The diluted antisera kept at room temperature for 15 minutes before incubating with NC strips for 4-6 hours at room temperature then overnight at 4°C. This was followed by four 8 minutes washes in TBS containing 0.05 Tween-20. The conjugate was diluted in TBS sera and incubated with NC strips for four hours at room temperature, followed by 4 washes as mentioned before and then a 3 minutes wash in TBS. Immunoblots were developed for 5 minutes using H_2O_2 and 4-chloro-1-naphthol then detected for specificity.

Evaluation of the recombinant protein

Evaluation of the recombinant protein was performed using antigen capture ELISA *(9)* as follows. Specific antisera (prepared against recombinant protein at the our lab.) were mixed with coating buffer to give a concentration of 1 ug/ml, then 100 ul of the preparation were distributed into the ELISA plate wells, and kept at 4°C for overnight. The excess capture solution was removed and washed with the PBS-Tween and repeated twice then excess solution was removed vigorously from wells onto tissue paper. 200 ul of PBS-BSA were added into each well to block the plate, then the plate sealed and kept at 37°C for 30 minutes. Blocking reagent was discarded and the plate washed as before. The control positive and control negative wells were determined 100 ul of the recombinant protein were added into the remaining wells in duplicate, then the plate was sealed and kept at room temperature for two hours. The plate then washed as before and 100 ul of antibody dilution were added, then plate was sealed and kept at room temperature for 2 hours. Then the plate was washed again and 100 ul of antibody conjugated preparation were added to the wells and kept at room temperature for 2 hours. The plate was washed and 20 ul of enzyme substrate (OPD "Orthophenylene-Diamine dihydrocgloride) was added to each well and kept in the dark and read after 3 minutes, the reaction stopped with 50µl of stopping solution to prevent further enzymatic activity till reading (absorbance reading at 405 nm).

Comparison of recombinant protein, *M. gallisepticum* whole cell sonicated antigen and imported ELISA coated antigen .

Using serum samples collected from experimentally infected chickens as follows. A total of 350 Hy-line chickens, 5 weeks old, originated from a flock serologically free from *M. synoviae* and *M. gallisepticum*. To confirm their serological status, sera were retested with both SPA and HI tests and found to be negative. Chickens were subdivided into 7 groups equally, 5 groups to be infected with *M. gallisepticum* strains, the 6th group was infected with *M. synoviae* strain and the rest group was kept as non-infected control negative group. Chickens were infected with 24 hours old broth culture containing 2.1 x 10^{10} CFU/ml of the mycoplasma strain. Each chicken was injected with 0.1 ml in the abdominal air sac, 0.1 ml intranasally and 0.1 ml intratracheally. Each group was kept separately in separate building and samples were randomly collected Table, 3.

RESULTS AND DISCUSSION

In the present work, using the M9 gene primers for the different local field isolates, PCR succeeded to amplify a DNA fragment corresponding to M9 gene and the PCR product appeared to be around 1 kb (999 bp) as shown in Fig. 1. A 999 bp band was generated by PCR using PCR primers based on the sequence of genomic DNA library *(10)*, and such band (999 bp) was responsible for encoding of the M9 protein.

Regarding the molecular cloning of M9 gene using the specific primers, the PCR product was ligated directly into a plasmid vector then transformed into competent cells of *E. coli*. The recombinant plasmids having correct inserts which validated by mini-preparation followed by digestion of recombinant plasmid by *SmaI* and *Nru* I enzymes as shown in Fig. 2. Then

electrophoresed in 0.7% agarose gels and it confirmed that the recombinant plasmids have correct inserts at the predetermined size, the vector alone was found to be at 3.33 kb while insert was around the 1 kb. Also, Puc 8 plasmid was used in cloning the total DNA from the vaccinal F-strain and the reference S6 strain and were identified by cloning and Southern hybridization analysis *(11)*. Moreover, PCR product was used in cloning and the procedure was confirmed by nucleotide sequencing analysis of the cloned PCR product *(12)*.

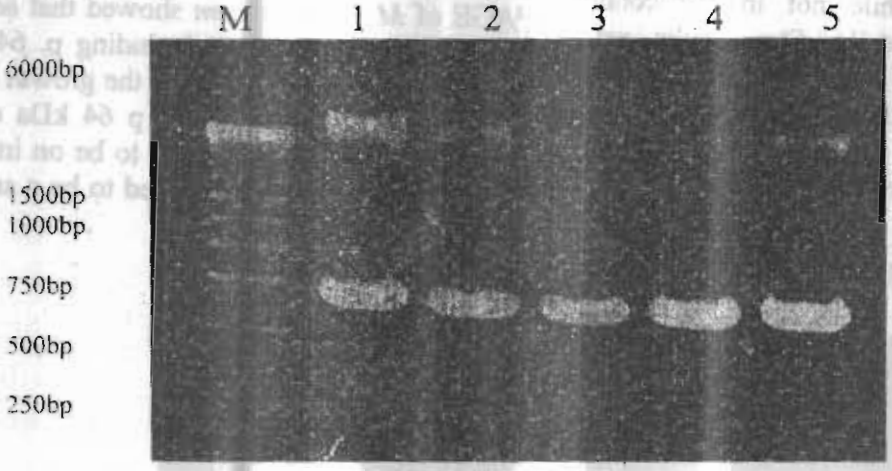

Figure 1: Agarose gel electrophoresis of M-9 gene PCR product from different isolates of M. gallisepticum (lane 1 to 5) , DNA ladder Marker (lane M)

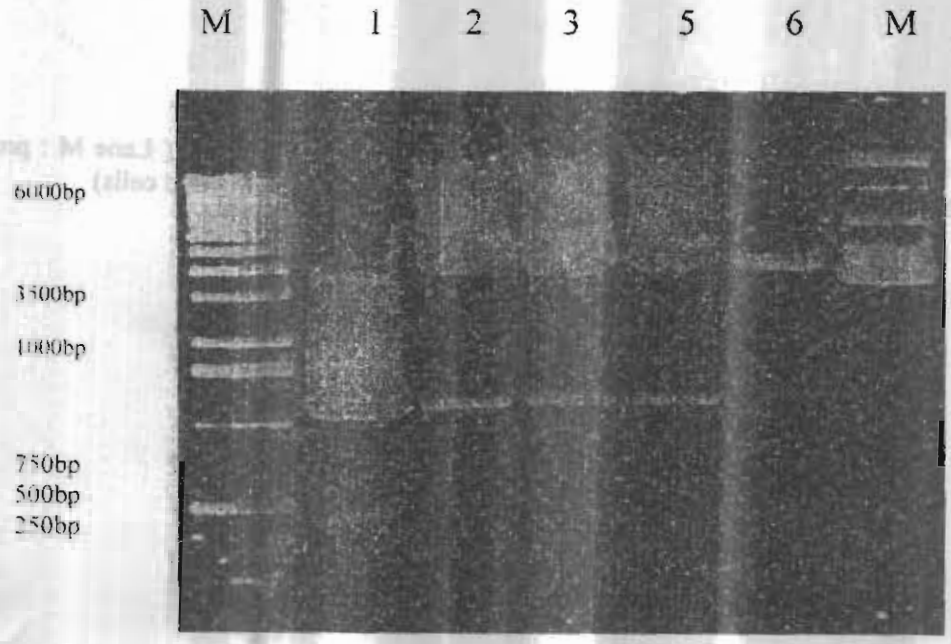

Figure 2: Restriction enzyme digestion of recombinant plasmid vector using SmaI and Nru I (DNA marker M, PCR product lane 1, plasmid alone after restriction digestion lane 2,3and 4 and plasmid containing the insert lane 6)

On looking to the SDS-PAGE of the protein profile of the recombinant cells as shown in Fig. 3, revealed a marked difference in the electrophoresed protein of 64 kDa molecular weight level of which present only in the recombinant cells while not in the control negative competent cells. Our results were previously confirmed as M9 gene has been shown to be expressed as surface protein in *M. gallisepticum* and it is detected in the cell lysates as 64 kDa protein bands ***(10)***.

Regarding the Western blot analysis for such protein it showed specific reactivity against specific *M. gallisepticum* antisera as shown in Fig. 4. Using immunoblotting ***(13)***, six immunogenic species specific proteins of *M. gallisepticum* were identified with relative molecular masses of 82 kDa, 64 kDa, 56 kDa, 35 kDa, 26 kDa and 24 kDa. Moreover, SDS-PAGE of *M. gallisepticum* showed that several *M. gallisepticum* proteins including p. 64 kDa and the anti-p64 IgG inhibited the growth of *M. gallisepticum* strains ***(2)***. So, p 64 kDa of *M. gallisepticum* has been shown to be on integral membrane protein and appeared to be a surface exposed protein.

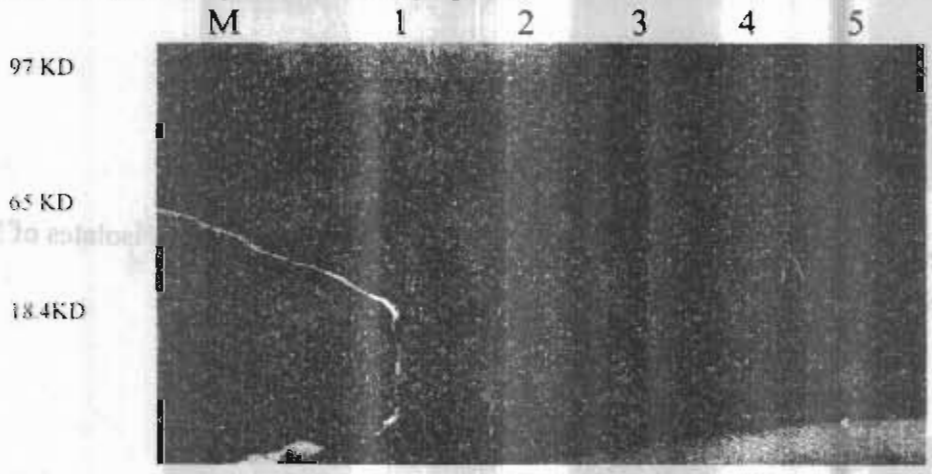

Figure 3: SDS-PAGE of *E. coli* strain JM 109 after expression showing M9 protein (Lane M : protein marker, Lane 1 and 2 : recombinant cells & Lane 3, 4 and 5 control noninduced cells)

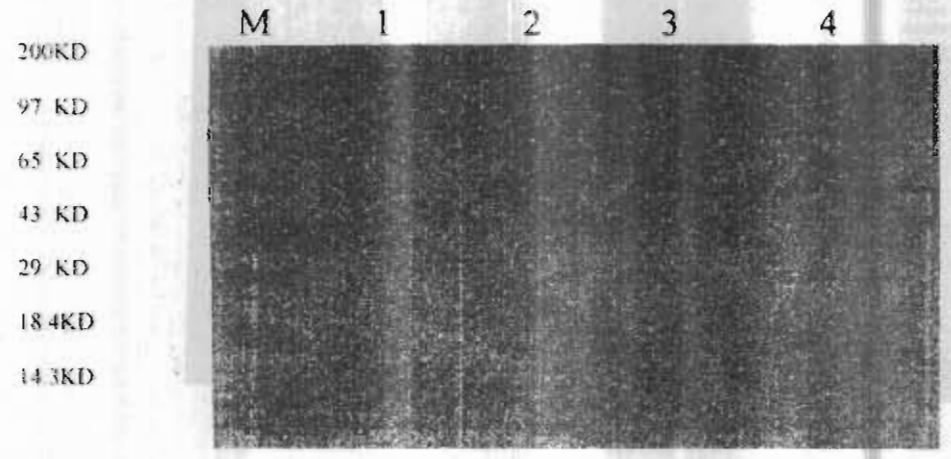

Figure 4: Western blot analysis of M9 protein reacted with polyclonal M. gallisepticum antisera (LaneM : prestained protein marker, Lane 1:noninduced cells, Lane 2, 3 and 4 recombinant cells).

The absorbance reading at 405 nm in ELISA for all positive sera were 2.5 times higher than the reading of the negative sera at the same dilution *(14)*.

As regard to the recombinant protein when used as ELISA antigen, the present results as illustrated in Table 1 showed that the realibility of such protein was started as early as 6th day post infection in only 2 out of 8 serum samples related to chicken groups infected with S6 and F-strain while in 9 out of 24 serum samples related to chicken group infected with the three different local isolates.

On the 9th day post infection, the activity of this protein appeared in all infected chicken groups in 50% with sera of chickens infected with S6 strain, 37.5% with sera of chicken group infected with F-strain and 50% with total sera of chicken infected with the tested local field isolates. Such activity increased gradually till reached its maximal level (100%) with all infected chicken groups on the day 43 post infection. These results nearly agreed with that described by previous investigators *(15)* reporting that ELISA titres were positive in all sera after 10 days and the mean ELISA titres reached their maximum on the 49th post challenge. Moreover, the antibody response to *M. gallisepticum* infection detected by ELISA was low at the 2nd week post infection and increased gradually to reach 80-100% at the 6th week post infection *(16)*.

Table 1. Comparison between ELISA positive values of the recombinant protein as ELISA antigen (> 0.294 absorbance units at 405 nm) against different *M. gallisepticum* antisera.

Chicken group ⇨	S6		F		Local Isolates					Negative control	
Days post infection ⇩	No. of +ve / Tested	%	No. of +ve / Tested	%	LI No. of +ve / Tested	LII No. of +ve / Tested	LIII No. of +ve / Tested	Total +ve/ Total tested	%	No. of +ve / Tested	%
3	0/8	0	0/8	0	0/8	0/8	0/8	0/24	0.0	0/8	0
6	2/8	25.0	2/8	25	4/8	2/8	3/8	9/24	37.5	0/8	0
9	4/8	50	3/8	37.5	4/8	4/8	4/8	12/24	50.0	0/8	0
12	5/8	62.5	4/8	50	6/8	4/8	5/8	15/24	62.5	0/8	0
15	5/8	62.5	5/8	62.5	6/8	5/8	6/8	17/24	70.8	0/8	0
22	6/8	75	5/8	62.5	6/8	6/5	6/8	18/24	75.0	0/8	0
29	6/8	75	6/8	75	7/8	6/8	7/8	20/24	83.3	0/8	0
36	7/8	87.5	7/8	87.5	7/8	7/8	7/8	21/24	87.5	0/8	0
43 and 56	8/8	100	8/8	100	8/8	8/8	8/8	24/24	100	0/8	0

S6: Chicken group infected with M. gallisepticum S6 strain.
F: Chicken group infected with M. gallisepticum F strain.
LI: Chicken group infected with M. gallisepticum local field isolate No. I.
LII: Chicken group infected with M. gallisepticum local field isolate No. II.
LIII: Chicken group infected with M. gallisepticum local field isolate No. III.
Negative control: Non infected control chicken group.

As regards to the correlation between the recombinant protein as ELISA antigen, sonicated *M. gallisepticum* antigen and imported ELISA coated antigens Table 2, the present study revealed that the sonicated antigen was sensitive than the imported one and finally there was the recombinant protein antigen especially at the first week post infection. This may be due to the differences in the strain and the method used in the preparation of antigen.

Table 2. Comparison of different ELISA antigens for detection of different M. gallisepticum antibodies.

Type of antigen ⇨	Recombinant protein		MG whole antigen		Imported antigen	
Days post infection ⇩	No. of +ve/ Total	%	No. of +ve/ Total	%	No. of +ve/ Total	%
3	0/24	0	1/24	4.2	1/24	4.2
6	7/24	29.2	9/24	37.5	8/24	33.3
9	11/24	45.8	11/24	45.8	13/24	54.2
12	14/24	58.3	13/24	54.2	15/24	62.5
15	16/24	66.7	15/24	62.5	17/24	70.8
22	17/24	70.8	17/24	70.8	18/24	75
29	19/24	79.2	20/24	83.3	20/24	83.3
36	21/24	87.5	21/24	87.5	21/24	87.5
43-50	24/24	100	24/24	100	24/24	100

As shown in Table 3, there is some variation between the three types of ELISA antigens against randomly taken serum samples where all antigens gave 100% reactivity with serum of chickens vaccinated with inactivated vaccine compared with 95.4%, 92% and 94.3% in using sonicated antigen, recombinant protein antigen and imported coated antige respectively on testing against serum of chickens vaccinated with live F-vaccine. Also, there is a clear variation in reactivity against serum samples taken from the unvaccinated chickens. Generally, the whole antigen showed 82.8% reactivity (232 out of 280 serum samples) compared with 81.8% reactivity for the imported coated antigen (229 out of 280) and only 79.6% reactivity for the recombinant protein (223 out of 280). Sera from naturally infected birds used for detection of *M. gallisepticum* in poultry showed that ELISA was more sensitive than HI test ***(14)***. The *M. gallisepticum* ELISA antigen followed the serum plate agglutination in sensitivity and the ability to detect the antibodies formed in early response to *M. gallisepticum* infection ***(17)***.

Table 3. Comparison of different ELISA antigens using randomly taken serum samples (Positive values > 0.294 absorbance units at 405 nm).

Type of antigen	Type of bird examined		No. of samples examined	No. of positive samples	% of positive samples
MG whole antigen	Vacc.	L	88	84	95.4 %
		D	69	69	100 %
	Non Vacc.	B	75	43	57.3 %
		La	48	36	75 %
	Total		280	232	82.8 %
Recombinant protein	Vacc.	L	88	81	92.0 %
		D	69	69	100 %
	Non Vacc.	B	75	39	52 %
		La	48	34	70.8 %
	Total		280	223	79.6 %
Imported ELISA coated plates	Vacc.	L	88	83	94.3 %
		D	69	69	100 %
	Non Vacc.	B	75	41	54.7 %
		La	48	36	75 %
	Total		280	229	81.8 %

MG: M. gallisepticum. Vacc.: Vaccinated. L: Birds vaccinated with live F-strain vaccine.
D: Birds vaccinated with inactivated S6 strain vaccine. B: Broiler birds not vaccinated.
La: Layer birds not vaccinated.

Table 4. Comparison between different types of M. gallisepticum ELISA antigens: Cross reactivity of M. synoviae antisera.

Type of antigen ⇨	MG whole antigen		Imported antigen		Recombinant protein	
Days post infection ⇩	No. of +ve / Total	%	No. of +ve / Total	%	No. of +ve / Total	%
3-12	0/8	0	0/8	0	0/8	0
15-22	1/8	12.5	0/8	0	0/8	0
29-36	2/8	25	1/8	12.5	0/8	0
3-50	2/8	25	2/8	25	0/8	0

The comparative study of specificity of the different three antigens through detection of its cross reactivity with *M. synoviae* antisera is illustrated in Table 4. The sonicated antigen started to react with *M. synoviae* chicken antisera as early as 15 days post infection in 12.5% of serum samples and reached its highest cross reactivity at the day 29 post infection in 25% of serum samples and remained till the end of experimentation compared with 12.5% at the 29th day post infection and reached 25% at the 43rd day post infection for the imported coated antigen against *M. synoviae* chicken sera. While in case of the recombinant protein showed no cross reactivity. These results confirmed that obtained by **Ansari et al.** ***(14)*** who reported that the positive sera for organisms other than *M. gallisepticum* and M. synoviae showed no reactivity to *M. gallisepticum* antigen. Also, it has been reported that the cross reactivity using sera from *M. synoviae* infected birds was obtained with ELISA in a few scattered cross reactions but the incidence never increased ***(18)***.

Moreover, ***Avakian and Kleven (13)*** confirmed the specificity of the recombinant protein and reported that 64 kDa protein is one of six species specific proteins of *M. gallisepticum*.

ELISA has been developed for the diagnosis of avian mycoplasma infection ***(19)***, but the commercial kits did not show the specificity and sensitivity desired for routine serological diagnosis of *M. gallisepticum* infection ***(20)***.

ELISA has been recently adapted for the diagnosis of several pathogens in poultry. Since repeated serological monitoring of flocks for a variety of infections is routine in health programming, it would be advantageous to employ the same methodology, such as the ELISA, for all infections, including mycoplasma. ELISA has been tried for the serological diagnosis of *M. gallisepticum*, but it showed a level of cross reactivity between *M. gallisepticum* and *M. synoviae* antibodies ***(21)***. So, among the numerous serological tests that have been developed, the interest in evaluating ELISA for more rapid, sensitive and specific detection of mycoplasma antibodies has been increased.

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الملخص العربى

استخدام البروتين M_9 كأنتجن للإليزا لتقييم الإستجابة المناعية لميكروب الميكوبلازما جاليسبتكم

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تم التأكد من العترات المعزولة محلياً من ميكروب الميكوبلازما جاليسبتكم باستخدام التجارب الكيميائية الحيوية البيوكيميائية والسيرولوجية، تم إجراء تحليل لها باستخدام الإنزيمات القاطعة التى أوضحت تباين بسيط بينها. بإجراء تفاعل البلمرة المتسلسل باستخدام بادئ حين الـ 16 rRNA وكذلك بادئ جين M_9 أوضح نتائج إيجابية لكلاهما مع كل المعزولات المحلية المختلفة. تم كلونة نتاج تفاعل البلمرة المتسلسل للجين M_9 داخل بلازميد وأدخل هذا البلازميد فى بكتيريا الإشريكية القولونية وتم الكشف عنه بطريقة المينى بريب المتبوع بالأنزيمات القاطعة وتم عمل الفصل الكهربائى للكشف عن البروتين المخلق وتم التأكد من وجوده باستخدام اختبار الويسترن بلوت. عند استخدام هذا البروتين كأنتيجين للأليزا أوضح حساسية أقل من الأنتجين المحضر من الخلايا الكاملة للميكروب وكذلك الأنتجين المستورد فى الأسبوع الأول بينما أوضح خصوصية عالية جداً يليه الأنتجين المستورد وكان الأنتيجين المحضر من الخلايا الكاملة لميكروب الميكوبلازما جاليسبتيكم هو أقلهم خصوصية.