Vet.Med.J.,57,No.2(2009) :235-242

EVALUATION OF THE RECOMBINANT M9 PROTEIN AS HAEMAGGLUTINATING ANTIGEN FOR SERODIAGNOSIS OF M. GALLISEPTICUM IN CHICKENS.

Salama S.S. ; Badawi A.A.;Zaki F.F. and Elham A. El-biary .
Central Laboratory for Evaluation of Vet. Biologics, Abbasia, Cairo.

Recived 23/08/ 2009 .
Accepted 15/09/2009 .

SUMMARY

This study focused on a prominent haemagglutination feature of the pathogenic *M. gallisepticum*, which have agglutinins that are immunogenic surface protein. M9 protein was prepared using cloning and expression technology and evaluated as haemagglutinating antigen. In comparing its haemagglutination properties with the whole cell antigen , the M9 protein showed somewhat less sensitivity starting after the first week post infection with *M. gallisepticum* strains and reaching the maximum reactivity at the seventh week post infection, while the whole cell antigen reached its maximum reactivity at the sixth week post infection. Regarding the specificity, both antigens are highly specific and showed no reactions with the *M. synoviae* antisera. The advantages of M9 protein as haemagglutinating antigen is that it could be used in different areas and is cheaper in preparation.

INTRODUCTION

Despite advances in diagnosis and treatment of infectious diseases , pathogenic microorganisms remain the most important threats to poultry production. Mycoplasma infection is still one of the most important respiratory disease of poultry, which when complicated with other pathogens results in the so known Chronic Respiratory Disease. This infection has a great economical importance due to poor growth gain, poor feed conversion, low grade carcass quality in broiler as well as lowering in egg production in laying birds ***(David and Avakian, 1992)***.

Thus it is necessary to have an effective and reliable tool for confirming mycoplasma infection in order to control and eradicate infection in poultry flocks. It is very efficient in detecting infected chickens even in the absence of symptoms ***(Abdel Moumen and Roy, 1995)***.

A major plasma membrane protein, pMGA, of *M. gallisepticum* has been identified as a cell

adhesion and/or haemagglutinin molecules and M9 protein is expressed by a prominent gene of pMGA gene family and is responsible for agglutination of the *M. gallisepticum* organisms ***(Li et al., 1998)***. So the objective of this study was to evaluate the recombinant M9 protein in haemagglutination inhibition test for the reliable detection of *M. gallisepticum* infection in poultry flocks.

MATERIAL AND METHODS

(1) Strains :

Three reference strains of *M. gallisepticum* (S6, R, and F) , one local field isolate of *M. gallisepticum* and one local field isolate of *M. synoviae* were used in this experiment.

(2) Whole *M. gallisepticum* haemagglutination antigen:

The antigen was prepared as mentioned by ***USDA (1984)*** using the local field isolate. The stock antigen was dispensed in 2.0 ml aliquots and stored at -80 C° until used.

(3) *M. gallisepticum* M9 recombinant protein:

The protein was prepared as previously mentioned by ***Salama et al. (2005)*** where the amplified PCR product of the specific M9 gene were cloned using the Pin point Xa1–T-vector system (Promega corporation, USA) , Transferred into JM109 competent cells (Promega ,USA) and the recombinant protein was purified by Pin point Xa protein purification system (Promega, USA). Finally the purified protein was characterized by using western blot analysis as described by ***Garcia and Kleven (1993)***.

(4) Evaluation of the recombinant M9 protein as haemagglutinating antigen:

Firstly the haeagglutinating power of the purified M9 recombinant protein was performed according to the directions of ***USDA (1984)*** , then the protein was applied in the proper haemagglutination inhibition test using serum samples collected from chicken (H & N serologically mycoplasma free) experimentally infected with S6, R and F strains of *M. gallisepticum* and so those infected with one local field isolate of *M. gallisepticum* and chicken group infected with local field isolate of *M. synoviae* (***Czifra et.al. 1993***).

The examined serum samples were collected from different infected chicken groups weekly up to 8th week post-infection. Also random serum samples collected under the field condition from commercial flocks either vaccinated or unvaccinated , broiler or layer flocks were included in the study.

(5) **Comparison of the M9 recombinant protein and Whole *M. gallisepticum* cell antigen as haemagglutinating agents:**

The same procedures mentioned above were performed using the *M. gallisepticum* whole cell antigen.

RESULTS AND DISCUSSION

Mycoplasma species that are pathogenic for poultry haemagglutinate erythrocytes and haemagglutination inhibition tests are used to determine titers of antibodies to *M. gallisepticum*, *M. synoviae* or *M. meleagridis* in serum samples ***(Kleven, 1997)***.

In the present study, the sensitivity of the recombinant M9 protein of *M. gallisepticum* as haemagglutinating antigen , started after the first week post-infection with only one serum samples related to chicken group infected with S6 *M. gallisepticum* strain, raised to three positive samples after the second week representing 37.5 % as shown in Table 1while the other group showed a lower level at the same intervals.

Table (1): Reactivity of M9 recombinant protein as haemagglutinating antigen against different *M. gallisepticum* antisera in the HI test.

Chicken group	S6		F		R		Local isolate		Total tested		Negative control	
Weeks post infection	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%
1	1/8	12.5	0/8	0.0	0/8	0.0	0/8	0.0	1/32	3.125	0/8	0.0
2	3/8	37.5	1/8	12.5	2/8	25.0	2/8	25.0	8/32	25.0	0/8	0.0
3	5/8	62.5	4/8	50.0	4/8	50.0	4/8	50.0	17/32	53.125	0/8	0.0
4	6/8	75.0	5/8	62.5	6/8	75.0	5/8	62.5	22/32	68.75	0/8	0.0
5	8/8	100.0	5/8	62.5	7/8	87.5	6/8	75.0	25/32	81.25	0/8	0.0
6	8/8	100.0	7/8	87.5	8/8	100.0	8/8	100.0	31/32	96.875	0/8	0.0
7	8/8	100.0	7/8	87.5	8/8	100.0	8/8	100.0	32/32	100.0	0/8	0.0
8	8/8	100.0	8/8	100.0	8/8	100.0	8/8	100.0	32/32	100.0	0/8	0.0

The total reactivity started as one positive sample out of 32 after the first week post-infection representing 3.125 % and the detected positive titer was gradually increased as shown in Table (1) till reaching 100 % reactivity 7 weeks post-infection. ***Li et al. (1998)*** reported that M9 protein of *M. gallisepticum* is a member of the pMGA family and its surface protein iduced agglutination. This is confirmed by ***Li et al. (2000)*** , who stated that *M. gallisepticum*

possesses a family of M9/ pMGA genes encoding an adhesion protein associated with haemagglutination. Also ***Baseggio et al. (1996)*** and ***Markham et al. (1993)*** indicated that the genetic determinants that code for the haemagglutinin are organized into a large family of genes but only one of these genes is predominately expressed in any given strain of *M. gallisepticum*. ***Vardaman and Yoder (1970)*** assayed that the HI test for *M. gallisepticum* used in serum samples from experimentally infected chickens raised on isolation cabinets.

On the other hand , on using the *M. gallisepticum* whole cell antigen as haemagglutinating agent revealed a relatively more rapid reactivity starting with four positive reactions out of thirty two serum samples after the first week post-infection raised to twelve positive serum samples representing 37.5 % after the second week post infection and the number of positive serum samples increased gradually reaching its maximum reactivity after the 6th week post-infection (Table 2). These results are coordinated with that mentioned by ***Timms and Cullen (1972)*** whose reported that the positive HI reaction occurred two weeks post-infection and all sera were showed positive titers at the 6th week post-infection .

Table (2): Reactivity of *M.gallisepticum* whole cell antigen as haemagglutinating antigen against different *M. gallisepticum* antisera in HI test.

Chicken group	S6		F		R		Local isolate		Total tested		Negative control	
Weeks post infection	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%	No +ve / tested	%
1	1/8	12.5	0/8	0.0	1/8	12.5	2/8	25.0	4/32	12.5	0/8	0.0
2	3/8	37.5	2/8	25.5	3/8	37.5	4/8	37.5	12/32	37.5	0/8	0.0
3	5/8	62.5	4/8	50.0	5/8	62.5	6/8	62.5	20/32	62.5	0/8	0.0
4	6/8	75.0	5/8	62.5	6/8	75.0	7/8	87.5	24/32	75.0	0/8	0.0
5	7/8	87.5	6/8	75.0	7/8	87.5	8/8	100.0	28/32	87.5	0/8	0.0
6	8/8	100.0	7/8	87.5	8/8	100.0	8/8	100.0	32/32	100.0	0/8	0.0
7	8/8	100.0	7/8	87.5	8/8	100.0	8/8	100.0	32/32	100.0	0/8	0.0
8	8/8	100.0	8/8	100.0	8/8	100.0	8/8	100.0	32/32	100.0	0/8	0.0

When random serum samples collected under different field condition were examined with HI test using both antigens, the M9 recombinant protein give positive reaction with 248 serum samples out of 299 when used as haemagglutinating antigen, compared with 255

positive serum samples when using the whole cell antigen representing 82.94 % and 85.28 % respectively (Table 3). These results may confirm data presented in Table (1) and Table (2) where the *M. gallisepticum* whole cell antigen showed a relatively higher sensitivity than the M9 recombinant protein when used as haemagglutinating agent in the haemagglutination inhibition test . This may be attributed to the fact that the whole cell antigen was prepared from the local field isolate of *M. gallisepticum* that carry all antigenic determinant under the Egyptian condition .This is supported by ***Kleven et al. (1998)*** who concluded that there may be some advantages in using haemagglutinating antigen prepared from local strain in areas tend to have recurring outbreaks.

Table (3): Comparison between M9 recombinant protein and M. gallisepticum whole cell as haemagglutinating antigen using randomly obtained chicken serum samples.

Type of antigen	Type of examined bird		No. of tested samples	No. of positive samples	%
M9 recombinant protein	Vaccinated	Live vaccine	65	59	90.76
		Dead vaccine	72	71	98.61
	Unvaccinated	Broiler	80	50	62.50
		Layer	82	68	82.92
	Total		299	248	82.94
M. gallisepticum whole cell antigen	Vaccinated	Live vaccine	65	61	93.84
		Dead vaccine	72	72	100.0
	Unvaccinated	Broiler	80	53	66.25
		Layer	82	69	84.14
	Total		299	255	85.28

Regarding the specificity of both antigens as shown in Table (4), it was found that neither the M9 recombinant protein nor the *M. gallisepticum* whole cell antigen showed positive reaction with the serum samples of chickens experimentally infected with *M. synoviae*. These results agrees with those reported by ***Vardaman and Yoder (1970)*** whose concluded that the haemagglutinating antigen of *M. gallisepticum* has good specificity in differentiating between *M. gallisepticum* and *M. synoviae* infections by HI procedure.

Table (4): Studying the cross reactivity against *M. synoviae* antisera using M9 recombinant protein and *M. gallisepticum* whole cell antigen .

Type of antigen	M9 recombinant protein		M. gallisepticum whole cell antigen	
Weeks post infection	Positive / tested	%	Positive / tested	%
1	0/8	0.0	0/8	0.0
2	0/8	0.0	0/8	0.0
3	0/8	0.0	0/8	0.0
4	0/8	0.0	0/8	0.0
5	0/8	0.0	0/8	0.0
6	0/8	0.0	0/8	0.0
7	0/8	0.0	0/8	0.0
8	0/8	0.0	0/8	0.0

From the obtained results , the *M. gallisepticum* whole cell antigen showed a relatively higher sensitivity than the M9 *M. gallisepticum* protein, while both are highly specific and showed no cross reaction with *M. synoviae* antisera. On the other hand the M9 protein is considered more cheaper in preparation and suitable for usage in different regions.

REFERENCES

Abdel Moumen ,B.B. and Roy R.S. (1995) : An enzyme linked immunosorbant assay for detection of Mycoplasmas in culture . Avian Dis., 39: 85 – 93 .

Baseggio N. ; Glew M.D.; Markham P.P.; Whithear K.G. and Browning G.F. (1996): Size and genomic location of the pMGA multigene family of M. gallisepticum . Microbiology 142: 1429 – 1435.

Czifra, G.; Sundquist B.G., Tuboly T. and Stipkovits L.(1993): Evaluation of monoclonal blocking ELISA for the detection of M. gallisepticum specific antibodies. Avian Dis., 37: 680 – 688 .

David H.L. and Avakian A.P. (1992) :An outbreak of M. synoviae infection in North Carolina Turkeys : Comparison of isolates by SDS-PAGE and Restriction endonuclease analysis. Avian Dis. , 36: 672 – 678.

Garcia, M. and Kleven ,S.H. (1993): Expression of M. gallisepticum F-strain surface epitope. Avian Dis., 37:494 – 500 .

Kleven S.H. (1997): Mycoplasma synoviae infection in Calnek B.W., Barnes H.J., Beard C.W., McDougald L.R. and Saif Y.M. (eds.) , Disease of poultry 9th edn (pp. 220 – 228) Ames, IA: Iowa State Uni. Press.

Kleven S.H., Morrow C.J., and Whithear K.G. (1998): Comparison of M. gallisepticum strains by haemagglutination inhibition and restriction endonuclease analysis. Avian Dis., 15 : 291 – 298.

Li LIU ; Kevin Dybvig; Victor S. Panangala; Vicky L. Vansanten and Christopher T. French (2000) : GAA trinucleotide repeat region regulates M9/pMGA Gene Expression in M. gallisepticum. Infection and Immunity, 68(2) :871 – 876

Li LIU ; Michael P.; Vicky L. Vansanten ; Kevin Dybvig; and Victor S. Panangala (1998): A protein M9 associated with monoclonal antibody mediated agglutination of M. gallisepticum is a member of the PMGA family. Infect. . Immun. 66(11): 5570 - 5575.

Markham P.F., Glew M.D.; Whithear K.G. and Walker I.D. (1993): Molecular cloning of a member of the gene family that encodes pMGA , A haemagglutinin of M. gallisepticum. Infect. Immun. 61: 903 – 909 .

Salama S.S.; Elham A. El-Ebiary; Hashad M.E. and Abdel Aziz M.A. (2005): M9 protein as an ELISA antigen for evaluation of immune response to M. gallisepticum. Zag. Vet. J., 33(3) : 75 – 85 .

Timms , L.M. and Cullen G.A. (1972): Comparison efficacy of 4 M. gallisepticum strains as antigens in detecting heterologous infection. Res. Vet. Sci., 13: 523 – 528 .

U S D A (1984): Standard requirement for avian mycoplasma antigens : Publication 0 – 78 Animal and Plant Health inspection services, Veterinary Services Branch, USDA Hyattsvill, Maryland.

Vardaman T.H. and Yoder H.W.Jr (1970): Mycoplasma synoviae and M. gallisepticum infection: Differentiation by haemagglutination inhibition test . Poult. Sci. 49: 157 – 161.

الملخص العربى

تقييم بروتين إم٩ المخلق كأنتيجن للتلازن الدموى للتشخيص السيرولوجى لميكروب الميكوبلازما جاليسبتكم فى الدجاج.

سليم سليم سلامة ، أحمد عبداللطيف بدوى ، فريد فؤاد ذكى، إلهام عطا الإبيارى
المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية

ألقت هذة الدراسة الضوء علي ظاهرة التلازن الدموي لميكروب الميكوبلازما جاليسبتكم الضاري والحامل لبروتين الأجلوتينين الموجود علي السطح الخارجي للميكروب وله صفات مناعية. تم تحضير بروتين إم ٩ واستخدامه عن طريق كلونة الجين المسئول عنه والتعبير عنه بإستخدام بلازميد خاص وتم تقيمه كأنتيجين للتلازن الدموي. بمقارنة نتائج الانتجين المحضر من خلايا الميكروب الكاملة والبروتين إم ٩ وجد أن البروتين إم ٩ أقل حساسية من الأنتيجين الكامل ولكن بنسبة ضئيلة تبدأ بعد الأسبوع الأول من الإصابة بميكروب الميكوبلازما جاليسبتكم حتي تصل الي اقصاها في الأسبوع السابع من العدوي بينما الأنتيجين المحضر من خلايا الميكروب الكاملة وصل الي أقصاها في نهاية الأسبوع السادس بعد الإصابة. بالنسبة للخصوصية أبدى كلا الانتيجينين خصوصية عالية ولم يعطي أي منهم أي تفاعلات مع السيرم المضاد للميكوبلازما سينوفي. الميزات المتحصل عليها للبروتين إم ٩ عند استخدامه كأنتيجين للتلازن الدموي هو إمكانية إستخدامه في مختلف المناطق الجغرافية والوبائية وكذلك هو أقل تكلفة من حيث التحضير.