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Preparation and Evaluation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* Recombinant Vaccine

Sabry I. Eissa*
Animal Health Research Institute, Mycoplasma Department, Dokki, Giza

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Abstract

Mycoplasma gallisepticum has three virulence genes (*mgc1, mgc2* and *mgc3*), and *Mycoplasma synoviae* has adhesion gene (*vlhA*). These genes are responsible for adhesion and colonization of the respiratory tract of the host. Therefore, this study was concerned with preparation of recombinant vaccine from field isolates which was compared with membrane and whole cells vaccines. The virulence genes were cloned into plasmid vectors followed by transformation into *E. coli* BL-21competent cells for the expression of adhesion proteins. Experimental study was performed for evaluation of the prepared *M. gallisepticum* recombinant vaccine. ELISA results for Mycoplasma recombinant vaccines revealed that there was a significant difference between the vaccinated and non-vaccinated groups at $p< 0.05$ for serum and egg yolk. On the other hand, results for *M. synoviae* antibody titers had no significant difference between the Geometric mean titers(GMTs) of the vaccinated groups but overall vaccinated groups had significant ($p< 0.05$) high antibody titers than the un-vaccinated group in both serum and egg yolk. Concerning the detection of *mycoplasma* species by polymerase chain reaction (PCR) and culture, recombinant vaccinated group were negative from the first week post-challenge till the end of experiment. While membrane and whole cell vaccinated groups gave weak positive results for *M. gallisepticum* in PCR assay and positive for culture (20% for membrane group and 60% for whole cell group). On the other hand, *M. synoviae* was detected (20%) by culture at one and three weeks post challenge and weak positive results was obtained by PCR till the end of experiment. The obtained results indicated that the recombinant vaccine was effective for protection of vaccinated bird against Mycoplasma infection.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Cloning, Expression of adhesion proteins, Recombinant vaccine.

Introduction

Mycoplasma gallisepticum causes severe economic losses to the poultry industry. Considering that eradication through elimination of positive flocks is expensive, available vaccines do not protect against infection, and the disease is difficult to effectively treat, new alternatives are needed to control the disease [1].

M. synoviae and *M. gallisepticum* are avian pathogens that can both cause respiratory disturbances. In addition, *M. gallisepticum* infection can lead to egg production losses and *M. synoviae* causes articular troubles [1]. Mycoplasmas have oval, filamentous or flask shapes, and several pathogenic species display a prominent polar tip organelle or bleb structure that mediates attachment to the host target cells. With the aid of the products of the *gapA*(or *mgc1*) gene [2,3] and *crm*A or *mgc*3 [4]. This tip structure is hemispherical, around 800x1250 A in circumferences and composed of surface-exposed proteins, called adhesins or cytadhesions proteins. These adhesions promote the attachment of mycoplasma allowing the colonization of epithelial cell surfaces [5, 6].

M. synoviae is a pathogen associated with osteoarthritis, synovitis, and respiratory tract lesions of poultry [7].Cytadherence mediated by its primary adhesion *vlhA* that is a precursor to virulence. Posttranslational cleavage of full-length *vlhA* produces the peptides *MS*PA (carboxy-terminal portion of *vlhA*) and MSPB

***Corresponding author e-mail: (drsabryismail@gmail.com), Animal Health Research Institute, Mycoplasma Department, Dokki, Giza.**

(amino-terminal portion). Receptor binding and cytadherence are attributed to *MS*PA, while the function of *MS*PB remains undefined [8].

As a consequence, recombinant technology could significantly help to alleviate the above mentioned drawbacks, allowing the production of unlimited amounts of multiple and more specific antigens [9]. Although identification of immunogenic antigens and genetic manipulations of mycoplasma in general are more laborious than any other prokaryotic genome, it is vital to successfully express *M. gallisepticum* proteins in heterologous systems such as *E. coli* [10].

Therefore, the present work aimed to prepare *M. gallisepticum* and *M. synoviae* recombinant vaccine to control mycoplasma infection in layers and breeders.

Materials and Methods

Mycoplasma strains

Four *M. gallisepticum* and two *M. synoviae* field strains were used in this study under accession numbers KY421064, KY421066, KY421065, KY42106 for *M. gallisepticum* and KY421062 and KY421063 for *M. synoviae* in Genbank.

Reference strains: *M. gallisepticum* S6 and *M. synoviae* WVU 1853 kindly given by Prof. Dr. Steve Geary, Connecticut University, USA.

Polymerase chain reaction (PCR) Amplification assays:

PCR amplification assays were carried out using extracted DNA via DNA extraction kit(QIA Amp® DNA Mini Kit - Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and oligonucleotide primers targeting *gap*A, *mgc*2 and *crm*A genes of *M. gallisepticum*[10, 11] and *vlh*A gene of *M. synoviae* [12]. The primers (Table1) were synthesized by Macrogen, Company, South Korea. PCR was performed in a total reaction volume of 25µL containing 12.5 µL of i-pfu PCR master mix (iNtRON, Korea), 5. µL of DNA template, 1 µL of each forward and reverse primer (20 pmol) and 5.5 µL nuclease free water.

Table 1: ***M. gallisepticum and M. synoviae*** **target genes, primer sequences and amplified product sizes used in the study**

Mycoplasma spp.	Target gene	Primer sequence (5' – 3')	Expected product size (bp)
M. gallisepticum	*gap*A	F- *CACCGCCGGA TTG ATT TGT ATG	1511
		R- ACT TGT TTT GTG TTT CC	
	*crm*A	F –ACCAGGGGATCCGCTCCAGCACCAACTAAGAAAATTGA	3263
		R- GGGGGATCCCCTTATCGTAGAGAAGGGAGGT	
	*mgc*2	F-CACCGCTTTGTGTTCTCGGGTGCTA	824
		R-CGGTGGAAAACCAGCTCTTG	
M. synoviae	*vlh*A	F-CACCCTTACAAGTACGGTGTTAAGTCATC	1100
		R-CGTATTTACAGCACCAGTAGTAACT	

* Added sequences for Topoisomerase enzyme insertion in the Topo vector

Purification of PCR products and sequence analysis

PCR products of the target genes were purified using GeneJET Genomic DNA purification kit (cat. No. K0721, Lithuania). Six purified PCR products were sequenced in GATC Biotech Company using ABI 3730xl DNA sequencer. DNA sequences were compared with those available in NCBI databases by using BLAST. Nucleotide sequence analysis was performed by MEGA5 program, product version 5.1 (www.megasoftware.net). Nucleotide sequences were deposited in the GenBank under accession numbers KY421064, KY421066, KY421065, KY42106 for MG and KY421062 and KY421063 for MS.

Preparation of mycoplasma antigens

Mycoplasma antigens were prepared according to Frey *et al.* [13]. *M. gallisepticum*

and *M. synoviae* were grown in Frey's media for 48 hours and harvested by centrifugation at 14000 rpm for 20 min. The pellet was washed three times with phosphate buffered saline (PBS), pH 7.2, then re-suspended in PBS and the protein concentration was estimated as previously described [14].

Membrane protein extraction

It was carried out using ProteoJET, Fermentas, cat. No. 89842X according to the manufacturers' instructions.

Preparation of recombinant vaccine

The vaccine was prepared according to Griffin, 1949[15]. *M. gallisepticum* (*gapA*, *mgc2* and *crmA*) and *M. synoviae* (*vlhA*) purified PCR products were cloned into plasmid vector pLUG- Prime TA-cloning Vector Kit (cat.No.11063. iNtRON, South Korea), followed by transformation into *E .coli* cells TOP10 chemically competent *E. coli* (Invitrogen cat. No. C4040-06, USA) then purified with plasmid DNA purification kit (DNA-Spin Plasmid DNA purification Kit cat. No. 17097 iNtRON, South Korea) followed by transformation into *E. coli* (BL-21chemically competent cells cat.No.CD60–China) for gene expression.

Expression of fusion proteins were induced by the addition of 1 mmol/L IPTG on Lysogeny broth and incubated at 37◦C for 12 h, then purified on a Nickel – NTA column (HisPur Ni-NTA columns, Thermo scientific, cat. No. 88225). The purified proteins were used for vaccine preparation.

Vaccine evaluation

Determination of Hydrophilic-Lipophilic Balance (HLB) value of the oil emulsion according to Cessi and Nardelli [16] and WHO [17].

Physical evaluation

Emulsion stability test was conducted using specification [18]. The Viscosity testing was carried out according to Cessi and Nardelli [16]. In brief, each 100 mL of the vaccine containing 9.7 mL span 80 (Sigma), 59 mL paraffin oil (Sigma) as oil phase, 1.8 mL tween 80 (sigma) and 29.5 mL PBS (1mg/mL membrane protein) as aqueous phase to prepare the vaccine.

Sterility testing

It was applied according to the Code of Federal Regulations "9 CFR" for detection of Bacteria, Fungi and Mycoplasma contamination.

Safety and potency test:

The challenge test was carried out according to Nicholas *et al.* [18].

Experimental design

Fifty female 85 days old commercial layers (ISA-Brown) were housed in cleaned, fumigated and well ventilated rooms in the central laboratory, Animal Health Research Institute, Dokki, Giza, Egypt. They were acclimatized for 2 days before the onset of the experiment. Non-medicated feed and water were provided. All Institutional and National Guidelines for the care and use of animals were followed.

The birds were screened by ELISA for detection of specific antibody titer (*Mycoplasma gallisepticum* cat. No. 96-65334-*Mycoplasma synoviae* cat. No. 96-6536Synbiotics, USA) and PCR tests to confirm that the birds were control for avian Mycoplasma. The birds were divided into 7 groups (5 birds for each); vaccinated with (*M. gallisepticum* recombinant (*MG*R), *MG* membrane (*MG*M), *MG* with *M. synoviae* recombinant (*MS*R), *MG* plus *M. synoviae* membrane (*MS*M), *M gallisepticum* whole cell (*MG*W), *MG*W with a *M. synoviae* whole cell (*MS*W) and non vaccinated control group. All vaccinated groups were kept in isolated unit away from the vaccinated groups. Throughout the study, birds were provided with feed and water and vitamins. The vaccines were given at two weeks old, with the corresponding vaccine for each vaccinated group with first dose (100 µg/mL subcutaneous), then received the second dose two weeks later. After two weeks the second dose was applied with same route and dose. After two weeks, all the nine vaccinated groups were challenged with the corresponding hot field strain of *M. gallisepticum* and *M. synoviae* (10^6 CFU/mL). Every two weeks blood samples, Eggs and tracheal swabs were collected till the end of experiment.

Fifty samples from vaccinated birds and control negative were tested for detection of *M. gallisepticum* and *M. synoviae* antibodies using ELISA assay. Each tracheal swab was inoculated into Frey's broth and incubated at 37 ◦C for 24 h and cultured on Frey's agar for re-isolation of mycoplasma.

Statistical analysis

Two way ANOVA test was followed by the Duncan multiple range test to analyze data of ELISA test for each vaccinated group. By computing LSD (least significant difference) we could determine if there was a significant difference between groups according to IBM= SPSS 20 [19].

Results

PCR succeeded to amplify *gapA*, *crmA* and *mgc2* of *M. gallisepticum*; and the *vlhA* gene of *M. synoviae* isolated from pelleted cells of *M. gallisepticum and M. synoviae* field and reference strains. The PCR products gave the expected amplicon size as demonstrated in Figure (1). The different genes were purified and sequenced. The sequence analysis of the field strains were compared with the reference strains on GenBank Data base and showed 100% similarity.

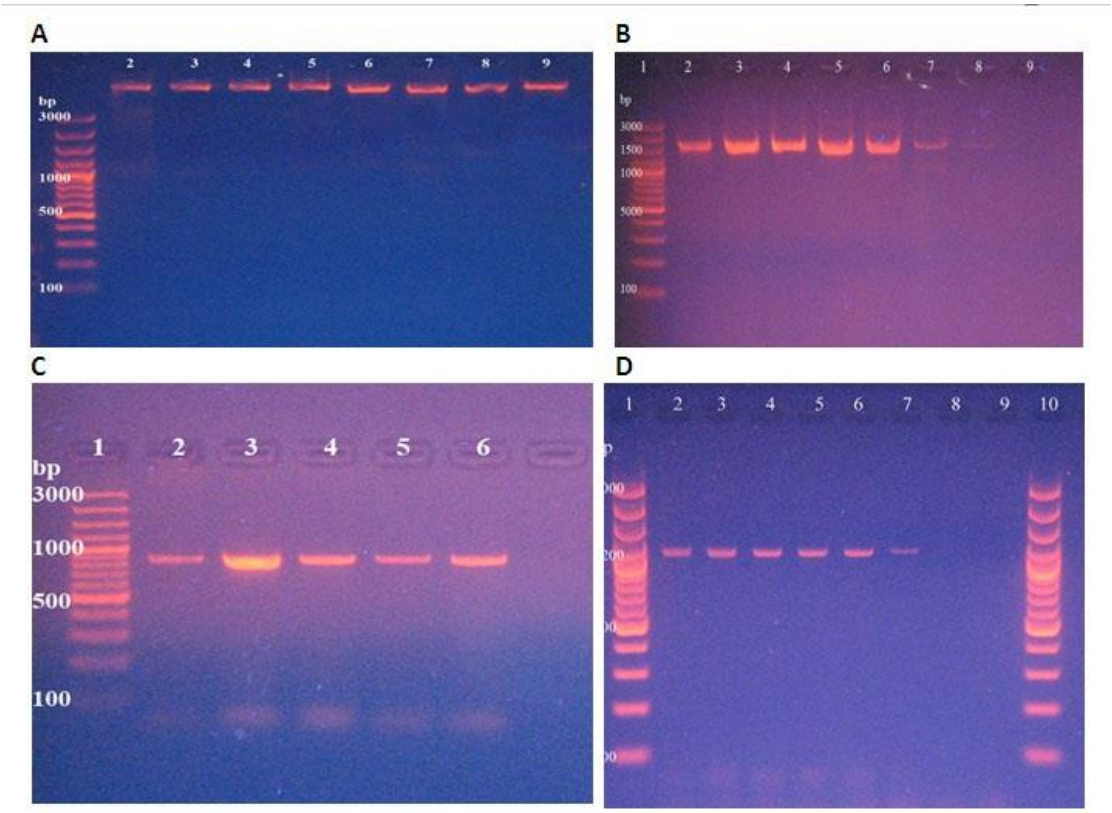

Figure 1: **(A):Agarose gel electrophoresis of PCR amplified product of *crmA* gene in *M. gallisepticum* field and reference strains. Lane 1: 100 bp DNA ladder; Lane 2: reference strain; Lanes 3-9: field isolates. (B): Amplified product of *gapA* gene of *M. gallisepticum* reference (S6) and field strain. Lane 1: 100 bp DNA ladder; Lane 2: reference strain; Lanes 3-9: field isolates. (C): Amplified product of *mgc2* gene of *M. gallisepticum* reference (S6) and field strains, Lane 1: 100 bp DNA ladder; Lane 2: reference strain; Lanes 3-6: field isolates. (D): Amplified product of *vlhA* gene of *M. synoviae* reference and field strains, Lane 1& 10: 100 bp DNA ladder; Lane 2: reference strain; Lanes 3-9: field isolates.**

Tracheal swabs that had turned yellow on Frey's broth were subjected to DNA extraction and tested by PCR test for the detection of *M. gallisepticum* and *M. synoviae* using specific primers.

As depicted in Table (2) there was significant difference between the means of the antibodies in serum samples of different vaccinated groups *MG* and *MS*R, *MG* and *MS*M and negative control group at $p<0.05$ and $p<0.01$. *MG*R, *MG*M, *MS* and *MG*W had

significantly higher means than control negative group at $p<0.05$.

For *M. gallisepticum* egg yolk there was significant difference between the means of the *MG* and *MS*R, *MG*W vaccinated groups and the non vaccinated birds at $p<0.05$ and $p<0.01$ but *MG*R, *MG*M, *MG, MS*M, *MS* and *MG*W had significantly higher means than control group at $p<0.05$.

For both serum and egg yolk, all vaccinated groups had significantly higher means than the non vaccinated group at $p< 0.05$.

Table (3) shows the geometric mean titers (GMTs) of *M. synoviae* in serum and egg yolk. The results proved that there were no significant differences between the GMTs of the vaccinated groups, but all vaccinated groups were significantly different from the control group at $p< 0.05$.

Table 2: Geomeric mean titers of *MG* ELISA in serum and egg yolk of different vaccinated and control groups of experiment

	Groups	2nd week	4th week	6th week	8th week	10thweek	12th week
Serum	MGR	832.5 ± 164.91 b	994.4± 187.33 b	1101.6± 185.29 c	1334.6± 206.38 c	1832.7± 325.95 d	1986.7± 354.26 d
	MGM	915.1 ± 169.24 b	1190.1 ± 198.54 c	1217.4 ± 223.87 c	1132.2 ± 215.47 c	1323.5± 216.88 c	1284.8 ± 248.39 c
	MG&MSR	1410.2± 223.76 c	1662.5± 289.35 d	1881.3 ± 342.58 d	1720.9± 339.24 d	2019.5± 362.98 d	2370.7± 374.85 e
	MG&MSM	1070.6± 164.22 b	1235.2± 218.94 c	1482.5± 237.83 c	1555.2± 254.28 d	1970.8± 332.52 d	2350.3 ± 374.12 e
	MGW	667.9 ± 115.29 b	845.2 ± 159.35 b	1202.4 ± 241.58 c	1075.8 ± 168.43 b	1145.3 ± 191.03 c	1289.1 ± 222.32 c
	MS&MGW	941.5 ± 166.43 b	1234.4± 254.68 c	1363.2± 251.38 c	1641.2 ± 300.68 d	1580.5 ± 314.58 d	1663.4± 325.98 d
	Control	48.09 ± 7.24 a	24.82 ± 4.86 a	59.9 ± 8.89 a	37.12 ± 6.67 a	43.51 ± 8.54 a	67.61± 11.21 a
Egg yolk	MGR	480.4 ± 106.31 b	740.3 ± 154.36 c	1009.4 ± 173.45 d	814.3 ± 159.52 c	1201.1± 194.67 e	1152.1 ± 180.61 e
	MGM	833.2± 155.42 c	886.5± 152.68 c	988.5 ± 177.14 c	968.9 ± 187.25 c	1079± 194.22 d	1221.2± 204.65 e
	MG&MSR	842.1 ± 151.47 b	1201.4 ± 211.03 e	1169.9 ± 189.51 e	1324.1 ± 209.66 e	1581.2± 251.96 f	1524.5± 254.34 f
	MG&MSM	660.9 ± 135.99 b	883.4± 151.22 c	1210.1 ± 189.33 e	1257.4 ± 193.21 e	1318.2± 202.54 e	1146.9± 180.67 e
	MGW	662.01± 128.73 b	1007.5± 168.24 d	1249.0 ± 212.89 e	1422.1± 232.14 f	1576.5 ± 256.69 f	1533.07 ± 261.87 f
	MS&MGW	262.1± 54.21 ab	638.7 ± 118.35 b	912.2± 169.47 b	1215.1 ± 182.55 e	1379.4 ± 197.03 e	1307.1 ± 213.41 e
	Control	55.66± 10.22 a	20.85 ± 3.87 a	32.91 ± 6.86 a	44.26± 9.47 a	36.34± 5.98 a	22.69± 4.16 a

MGR: *M. gallisepticum* recombinant, MGM: MG membrane, MG&MSR:MG with *M. synoviae* recombinant, MG&MSM: MG plus *M. synoviae* membrane, MGW: *M gallisepticum* whole cell, MS&MGW: *M. synoviae* plus *M gallisepticum* whole cell and non vaccinated control group. Means within the same column with different superscripts are significantly different (P<0.05).

Table 3: Geomeric mean titers of *M. synoviae* ELISA in serum and egg yolk of different vaccinated and control groups of experiment

Type of samples	Groups	2nd week	4th week	6th week	8th week	10th week	12th week
Serum	MSR	725.4± 152.65 b	951.6b± 164.38	1165.3 183.79 c	1684.4± 238.11 d	1986.2± 285.38 d	2114.5 ± 297.32 e
	MSM	954.6 ± 168.35 b	912.3 ± 167.24 b	1478.1 ± 224.11 c	2314.8 ± 286.37 e	2264.4 ± 281.44 e	2754.6 ± 357.24 f
	MG&MSR	972.7 ± 159.91 b	1055.6± 163.87 c	1285.7 ± 182.92 c	2008.6± 269.63 d	1948.3± 266.75 d	2338.1± 324.21 e
	MG&MSM	872.5 ± 158.35 b	982.6 ± 166.47 b	1604.6± 241.36 d	2476.8 ± 316.70 e	2854.9 ± 371.88 f	2908.7 ± 388.64 f
	MSW	667.6 ± 152.38 b	772± 156.74 b	1686.2± 229.64 d	2376.7 ± 294.79 e	2296.2 ± 271.34 e	2440.6 ± 296.41 e
	MS&MGW	822.3 ± 155.36 b	970.8 ± 160.21 b	1592.3 ± 231.10 d	2434.8± 301.59 e	2314.6 ± 281.55 e	1689.6± 234.47 d
	Control	48.09 ± 7.13 a	24.82± 5.22 a	59.9 ± 9.51 a	37.12 ± 6.12 a	43.51 ± 6.69 a	67.61 ± 7.74 a
Egg yolk	*MS*R	662.5 ± 137.77 b	810.4 ± 149.18 b	1020.8± 172.66 c	1656.4 ± 251.41 d	2015.3± 283.46 d	2116.5 ± 317.25 e
	*MS*M	447.1 ± 109.24 b	902.5 ± 157.28 b	1393.9 ± 213.87 c	2077.5 ± 292.35 d	2143.2 ± 327.22 e	2227.1± 351.25 e
	*MG&MS*R	522.3 ± 89.54 b	757.4 ± 117.20 b	1224.6 ± 174.56 c	1938.2 ± 279.35 d	1852.9 ± 282.21 d	2257.6 ± 270.55 e
	*MG&MS*M	641.2 ± 121.29 b	741.2 ± 133.84 b	1438.8 ± 251.32 c	2197.5 ± 345.52 e	2279.6 ± 362.14 e	2115.4 ± 321.47 e
	*MS*W	736.5 ± 141.25 b	811.7 ± 156.32 b	1422.3 ± 242.36 c	2228.4 ± 292.15 e	2146.2± 285.35 e	1960.4 ± 263.19 d
	*MS&MG*W	548.9 ± 103.54 b	849.2 ± 167.25 b	1506.2 ± 225.18 c	2132.2 ± 299.27 e	2017.5± 297.36 d	2054.1 ± 287.94 d
	Control	41.46 ± 4.25 a	35.53 ± 6.55 a	15.52 ± 3.27 a	51.4 ± 6.68 a	51.96 ± 9.42 a	37.24 ± 6.66 a

MGR: *M. gallisepticum* recombinant, MGM: MG membrane, MG&MSR:MG with *M. synoviae* recombinant, MG&MSM: MG plus *M. synoviae* membrane, MGW: *M gallisepticum* whole cell, MS&MGW: *M. synoviae* plus *M gallisepticum* whole cell and non vaccinated control group. Means within the same column with different superscripts are significantly different (P<0.05).

Concerning PCR and culture, *MG*R, *MG* and *MS*R vaccinated groups were negative from the first week post-challenge and continued till the end of experiment. While *MG*M, *MG* and *MS*M, *MG*W, *MG* and *MS*W vaccinated groups gave weak positive results with *M. gallisepticum* PCR (Figure 2) and positive for culture (20% for membrane group and 60% for whole cell group) .

On the other hand, *M. synoviae* PCR and culture were positive in *MS*M, *MS* plus *MG*M, *MS*W, *MS* and *MG*W vaccinated groups (Table3). While *MS*R, and *MS* with *MG*R vaccinated groups gave positive results (20%) by culture at one and three weeks post challenge and gave weak positive PCR results, a week post challenge and during all the experimental periods (Figure 2).

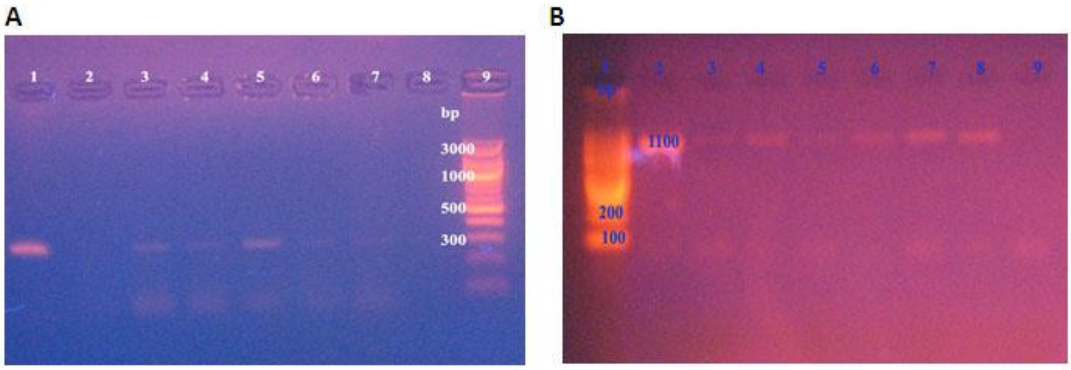

Figure 2: (A): PCR results of *M. gallisepticum* for different vaccinated groups of experiment; Lane1: Control positive, Lane 2: control negative; Lane 3: *MG*&*MS* whole cell bacterin; Lane 4: *MG* whole cell bacterin; Lane 5: *MG*&*MS* membrane; Lane 6: *MG and MS* recombinant; Lane 7: *MG* membrane; Lane 8: *MG* recombinant; Lane 9: 100 bp DNA ladder. (B): PCR results of *M. synoviae* for different vaccinated groups of experiment: Lane 1: 100 bp DNA ladder; Lane 2: Control positive; Lane 3: *MS* recombinant; Lane 4: *MS* membrane; Lane 5: *MG*&*MS* recombinant; Lane 6: *MG*&*MS* membrane; Lane 7: *MS* whole cell bacterin; Lane 8: *MG*&*MS* whole; Lane 8: *MG*&*MS* whole cell bacterin; Lane 9:non vaccinated group

Discussion

M. gallisepticum (R low passage strain) is pathogenic for chickens, colonizing the trachea (during *in vivo* assays) and producing air sac and tracheal lesions due to presence of *gapA* and *crmA* genes. In contrast, R high strains required 4 orders of magnitude for more organisms to colonize air sacs and tracheas to produce detectable lesions [20].Also it was mentioned that *mgc2* gene is incorporated in cytadherence, virulence and post infection autoimmunity[21]. For *M.synoviae*, it was reported that *vlhA* gene is a precursor of virulence [8]. In the present study we detected the three virulence genes of *M. gallisepticum* (*gapA*, *crmA* and *mgc2*) and *vlhA* gene of *M. synoviae* in our field strains. The purified genes were used for cloning in plasmid vector and expression in *E. coli* competent cells. The resulted recombinant proteins were used for preparation of recombinant vaccine. Also *MG* and *MS* membrane and whole cell bacterin were prepared. The obtained results proved that the recombinant vaccine can replace the other MG and MS vaccine due to good protection and healthy condition of vaccinated groups even after challenge with the virulent field strains. Besides the positive antibody titer two weeks post-vaccination and increased till the end of experiment. The results showed no significant differences among the vaccinated groups, while there was difference when compared with control negative group.

After challenge of the all vaccinated groups with MG and MS field strain, the recombinant vaccinated groups proved to be negative for PCR even at the end of experiment. On the other hand, membrane and whole cell vaccinated group showed positive PCR results.

In membrane vaccinated chickens, the antibody levels remained the same as after the challenge and the bacterin vaccinated group showed the highest titer during the entire experiment. This was found to be in agreement with Moura *et al*.[22] who mentioned that all vaccinated groups gave higher antibody titers and with El- Shater *et al*. [23] who concluded that vaccination of chickens with *M. gallisepticum* subunit vaccine resulted in high antibody response at two weeks after the booster dose.

Concerning the *MS* vaccinated groups; recombinant vaccinated groups gave weak positive results, while membrane and whole cell vaccinated groups were positive. Trials for re-isolation of Mycoplasma from different vaccinated groups after challenge were done. *MG* and *MG* and *MS* recombinant vaccinated groups and control group were negative during the experimental period, while membrane and whole cell vaccinated groups were positive, this coincides with El- Shater *et al.[*23]who concluded that *M. gallisepticum* subunit vaccine show humoral immunity but didn't give protection against infection, indicating

that Mycoplasmas may have ways to overcome humoral immunity.

Conclusion

The obtained results proved that the locally prepared *MG* and *MS* recombinant vaccine from local field isolates was the best choice for vaccination and protection of the commercial layers against mycoplasma infection in comparison with membrane and whole cell vaccines.

Conflict of interest

The author declares that he has no conflicts of interests.

Acknowledgement

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الملخص العربي

تحضير وتقييم لقاح مؤتلف للميكوبلازما جاليسبتكم والميكوبلازما سينوفي

صبرى اسماعيل عيسى

معهد بحوث صحة الحيوان - قسم الميكوبلازما - الدقى – الجيزة

تتضمن الميكوبلازما جاليسبتكم ثلاث جينات ضراوة (*mgc*1, *mgc*2, *mgc*3) بينما تتضمن الميكوبلازما سينوفي جين التصاق واحد (*vlh*A) . هذه الجينات مسئوله عن الالتصاق وتكوين مستعمرات الميكوبلازما علي القناه التنفسيه للعائل. ولهذا كانت هذه الدراسة عن تحضير لقاح مؤتلف من العترات الحقليه للميكوبلازما جاليسبتكم وسينوفي. تم استنساخ جينات الضراوة في ناقلات البلازميد ثم تحويلها في خلايا (*E.coli* BL-21) المؤهله وذلك للتعبير عن بروتينات الإلتصاق.تم عمل تجربه معمليه لتقييم اللقاحات المحضرة.بالنسبه للميكوبلازما جاليسبتكم كانت متوسطات المجموعات المحصنه باللقاح المؤتلف أعلي متوسط بفرق معنوي لكل من السيرم وصفار البيض باختبار الاليزا عن المجموعه السلبيه وذلك باحتمال اقل من ٠.٠٥ .بالنسبه للميكوبلازما سينوفي لم يكن هناك فرق معنوي بين المتوسطات للمجموعات المحصنه وكانت كلها أعلي بفارق معنوي عند أحتمال اقل من ٠.٠٥ من المتوسط الهندسي للمجموعه السلبيه وذلك في كل من السيرم وصفار البيض.فيما يتعلق بالعزل واختبار تفاعل البلمره المتسلسل وجد أن المجموعه المحصنه باللقاح المؤتلف للميكوبلازما جاليسبتكم قد أعطي نتائج ايجابيه ضعيفه باختبار تفاعل البلمره المتسلسل وايجابيه للعزل بنسبه(٢٠-٦٠%) وذلك بعد اسبوعين من العدوي وحتي اخر التجربه.من ناحيه أخري كانت نتائج العزل للميكوبلازما سينوفي ايجابية بنسبه٢٠% عند الاسبوع الاول والثالث بعد العدوي وحتي أخر التجربه.هذه النتائج اظهرت ان اللقاح المؤتلف أعطي نتائج مرضيه عن اللقاح الغشائي واللقاح الخلوي الكامل.