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AN APPROACH FOR IMPROVEMENT OF THE FOWL CHOLERA ADJUVANTED VACCINE IN EGYPT PRIVATE

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SUMMARY

Reformulation of the local oil adjuvanted fowl cholera inactivated vaccine components, the nature of adjuvant and vaccinal antigen, was approached aiming to improve its protective efficacy in chickens. A substantial value of reformulation was verified by protection against challenge exposure to both virulent serotypes A and D of *P. multocida* and seroconversion was detected by indirect ELISA and indirect haemagglutination (IHA) assays. All vaccinal antigens comprised formalized cultures of *P. multocida* serotypes A:5, 8, 9 and D:2 emulsified in the currently used water-in-oil adjuvant (Span, Paraffin and Tween-80, SPT80) and / or a commercial oil-in-water adjuvant (EMG). Besides, use of non-formalin treated lysates of *P. multocida* as a novel vaccinal antigen was evaluated in parallel. The oil-in-water adjuvant (EMG) was preponderant when used, with and without the currently used water-in-oil adjuvant (SPT80) in all new vaccine formulations, producing a stable, more potent, less viscous emulsion and easy injectable product. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis revealed a considerable similarity in the electrophoretic patterns of cell lysates recovered from *P. multocida* serotypes A:5, 8, 9 and D:2, manifested as 14-16 protein bands and the molecular mass range of about 14-190 kilodaltons. However, a considerable reduction in number of protein bands was observed in the cell lysates of their counterparts after formalin treatment. All new vaccine formulations used in this study are found reliable for seroconversion and protection of vaccinated chickens, with variable effectiveness. Use o f non-formalin treated lysates of *P. multocida* as a novel vaccinal antigen emulsified in EMG as an oil-in-water adjuvant, provided an advantageous formulation of the fowl cholera vaccine that conferred a 100% protection of chickens against virulent challenge. Moreover, it became much more safer vaccine for chickens since it contains a pharmaceutical grade

oil of EMG with no formalin residues. This work initiates a potential for production of safer bacterial vaccines for edible animals

INTRODUCTION

Fowl cholera (avian pasteurellosis) is one of the most devastating avian diseases that can affect all types of birds and is responsible for significant economic losses to poultry farms throughout the world (Derieux, 1978; Rhoades and Rimler, 1991). Fowl cholera may be caused by any of the 16 Heddleston serotypes of the bacterium Pasteurella multocida, although certain serotypes appear to be more often associated with disease (Heddleston et al., 1972). The disease is manifested usually as an acute septicaemia with high morbidity and mortality rates. It is often fatal however, mild and chronic forms of the disease may occur (Rhoades and Rimler, 1991).

Vaccination plays a main role in the control of fowl cholera. There are two types of fowl cholera vaccines, live attenuated and inactivated bacterin (Avakian et al., 1985). Inactivated vaccines gained much more interest than live vaccines due to safety concerns. However, inactivated vaccines may not confer full protection because of either a low antigen load or alteration of important epitopes during inactivation process and are generally poor cellular immunity inducers. The generally used *P. multocida* vaccines are bacterins, containing either aluminium hydroxide (Alls et al., 1969) or mineral oil as an adjuvants, prepared from inactivated whole bacterial cultures of serotypes selected on the basis of epidemiological information (Rimler, 1994).

In Egypt, the locally produced polyvalent formalin-inactivated water-in-oil adjuvanted bacterin, containing *P. multocida* serotypes A:5, A:8, A:9 and D:2, is currently used for immunization of chickens, ducks and turkey against fowl cholera (Azzam et al., 1992 and Gorgi, 1992).

Even though oil adjuvanted vaccine elicited a much more steady and stronger immune response than aluminium hydroxide adjuvanted vaccine, the water-in-oil adjuvants have poor syringeability, due to large amount of oil used, and may cause abscesses at the injection site. Antigenicity of a bacterial suspension is markedly increased when the bacteria are incorporated in an oil-in-water emulsion (Vanselow, 1987). Recently, some emulsified oil-in-water adjuvants, such as EMG, have been designed to be mixed directly with vaccinal antigens with reduced quantity of oil in the final product, resulting in decreased vaccine viscosity and minimal tissue irritation. These adjuvants are currently regarded as an excellent antigen delivery system for veterinary use.

Serologic characteristics of *P. multocida* are based on the antigenic properties of the capsular

and cell wall components (Manning, 1982). Thus, the immune response is directed mainly against the bacterial cell components not to the whole culture. It has been reported that formalin inactivation alter some of the proteinous vaccinal antigens and produce residual formalin byproduct in the meat of recipient animal (Monaco, 1992).

The objectives of this study were to: (1) investigate the efficacy of the oil-in-water adjuvants such as EMG in improving properties of the local inactivated fowl cholera vaccine, regarding the level of conferred protection and vaccine syringeability; (2) determine any possible change in the cellular protein profiles of the vaccinal serotypes of *P. multocida* due to formalin inactivation; and (3) verify the use of bacterial cell lysates of *P. multocida* as vaccinal antigens with neither formalin inactivation nor use of whole culture.

MATERIAL AND METHODS

1. Laboratory animals:

a. Chickens: A total of 130, one day old, chicks were obtained from the United Company for Poultry Production, assigned to 5 groups and reared under hygienic measures till reaching 6 weeks of age. They were screened for anti- *P. multocida* serotypes A and D antibodies using indirect haemagglutination test and all proved susceptible to fowl cholera (Carter and Rappy, 1962).

b. Mice: A total of 55 Swiss albino mice weighing about 18-20 g were used for safety of prepared vaccines (as 11 mice for each group).

2- Sera:

Antiserum: A polyclonal chickens antiserum was prepared against formalin-killed whole culture of *P. multocida* types A and D. It was used as a control positive serum for serological assays. It had an ELISA mean absorbance of 1.75 and 1.63 for types A and D, respectively.

Serum Samples: Chickens sera were collected from vaccinated and control chickens groups I, II, III, IV and V on weekly intervals as shown in Tables (1 and 2).

3. Bacterial strains: Four vaccinal bacterial strains of *P. multocida* serotypes (A:5, 8, 9 and D:2) were used for all vaccine preparations.

4. Bacterial culture inactivation: Bacterial strains were propagated separately in casamino acid medium (Bain, 1963) for 24 hours at 37°C with gentle agitation. Samples were taken to check purity and determine bacterial cell count per ml. The bacterial cultures were then inactivated for 24 hours at 37°C with 0.5% (v/v) formalin. The vaccine was standardized to contain 3×10^9 bacterial cells/ml.

5. Preparation of bacterial cell lysates: Bacterial cells of the four *P. multocida* serotypes were pelleted and washed twice in PBS (pH 7.4) with gentle handling. The washed cells were lysed in a lysis buffer, mixed in equal

volumes of each serotype and concentration of the lysate composite was adjusted to 75mg/ml (Brogden and Rimler, 1982).

6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Bacterial cell lysates of the four vaccinal *P. multocida* serotypes A:5, 8, 9 and D:2 before and after formalin treatment, were separated through vertical mini-slab (Mini-Protean II) gel electrophoresis cell, holding 10% separating gel and 5% stacking gel, following the standard procedures of Sambrook et al. (1989). After staining in 0.25% Coomassie Brilliant Blue R250 solution, the apparent molecular masses of separated components were estimated relative to their electrophoretic mobilities versus those of the pre-stained molecular weight protein standard, high range (14-200 kilodaltons, Gibco, BRL).

7. Preparation of adjuvanted fowl cholera vaccines:

A. Local conventional oil adjuvanted bacterin (Stone et al., 1978) produced at the Veterinary Serum and Vaccine Researches Institute, Abbassia, Cairo (VSVRI), containing formalin inactivated whole culture of *P. multocida* serotypes A:5, 8, 9 and D:2, emulsified in span, paraffin oil and Tween-80 (SPT80, water-in-oil adjuvant).

B. Modified oil adjuvanted bacterin containing formalin inactivated whole culture of *P. multocida* serotypes A:5, 8, 9 and D:2, emulsified in EMG (oil-in-water adjuvant) only according to the manufacturer instructions (MVP Laboratories, Ralston, USA).

C. Modified double oil adjuvanted bacterin containing formalin inactivated whole culture of *P. multocida* serotypes A:5, 8, 9 and D:2, emulsified in equal ratios of EMG and SPT80 according to the manufacturers instructions (MVP Labs and VSVRI).

D. Novel oil adjuvanted lysate vaccine comprising only non-formalized bacterial cell lysates of P. multocida serotypes A:5, 8, 9 and D:2 emulsified in EMG.

8. Quality control of the prepared vaccines: The prepared vaccines in this study were tested for sterility from any contaminant, safety in lab. mice and potency (seroconversion, challenge exposure to virulent *P. multocida* and lesion score in vaccinated chickens) following the standard international protocols as described by the Code of American Federal Regulation (1985).

9. Experimental design: Chickens were assigned to 5 groups, 26 birds each. They were vaccinated twice at 6 and 9 weeks of age with 0.5 ml a bird, for each vaccine, subcutaneously (S/C) in the middle part of the neck back as follows :

Group (I): Immunized with the modified fowl cholera formalin-inactivated, EMG adjuvanted bacterin.

Group (II): Immunized with the modified fowl cholera formalin-inactivated, double adjuvanted (SPT80 and EMG) bacterin.

Group (III): Immunized with the local conventional fowl cholera formalin-inactivated, SPT80 adjuvanted bacterin.

Group (IV): Immunized with the novel fowl cholera non-formalized lysates, EMG adjuvanted vaccine.

Group (V): The remainder 26 birds were kept as non-vaccinated control during the experimental period.

Challenge exposure: The protective efficacy of all vaccines used in this study was evaluated by virulent challenge exposure. Vaccinated and non-vaccinated control chickens were inoculated intramuscularly with 0.1 ml a bird, containing 10 lethal dose fifty (LD_{50}) of 24 hours-old culture of *P. multocida* serotypes A and D as was suggested by Heddleston and Rebers (1968). Clinical signs, mortality rates and gross lesions were recorded for the 14 successive days after challenge. Also, reisolation of viable *P. multocida* was tried from liver, blood and bone marrow of dead birds.

9. Serological investigations :

a. Indirect Haemagglutination (IHA): The capsular antigens of *P. multocida* types A and D were used in the IHA test to detect anti-*P. multocida* antibodies in chickens sera following the procedure described by Carter and Rappy (1962). Serum samples with a reciprocal haemagglutinating geometric mean antibody titre (GMT) less than 32 were considered negative.

b. Indirect Enzyme linked immunosorbent assay (ELISA): It was carried out following the procedure described by Voller et al. (1976), using sonicated antigens prepared from *P. multocida* serotypes A and D according to Brigg's and Skeels (1984). The 96-well microtiter plates (Nunc Immunoplates) were coated with 0.1ml of the sonicated (A and D separately) antigens (20 µg / well; dilution of 1:50), diluted in carbonate - bicarbonate coating buffer (pH9.6) and incubated at 4°C overnight. The microtiter plates were washed and blocked with 5% bovine serum albumin in phosphate buffer saline (PBS, pH7.4) for 1 hour prior to addition of 0.1ml of serum samples diluted 1:10 in PBS (pH7.4), in duplicates for each sample. Each microtiter plate contained positive and negative sera as well as a blank as controls. After 2 hours incubation at 37°C and washing of plates in PBS (pH7.4), a 0.1ml/well of the horse radish peroxidase labeled anti-chickens IgG whole molecule conjugate (Sigma) was added and allowed to react for 1 hour at 37°C. A 50 µl / well of freshly prepared substrate solution (Orthophenylenediamine, OPD, sigma) was added and the plates were incubated at 37°C for 20 minutes, with gentle agitation. Reaction was stopped with 25 µl / well of 1.25 M sulphuric acid and optical denisities (ODs) were read at wavelength 492 nm, using the microplate ELISA auto reader (Dynatech). The cut off mean absorbance value was 0.29 and 0.24 for serotypes A and D, respectively. Above these cut off values, a serum sample was regarded as positive.

RESULTS

EMG (oil-in-water adjuvant) was directly added to the vaccinal antigen component, forming smooth uniform emulsion with no need for emulsifying equipment currently used with water-in-oil adjuvants. Also, it made syringeability of the final products (vaccines B, C and D) a lot easier during vaccination compared to the currently used water-in-oil (SPT-80) adjuvant (vaccine A).

SDS-PAGE analysis revealed a considerable similarity in the electrophoretic patterns of cell lysates recovered from P. multocida types A:5, 8, 9 and D:2, manifested by 14-16 protein subunits at the molecular mass range of about 14-190 kilodaltons (kDa). However, a significant reduction in number of protein subunits was observed in cell lysates of their counterparts after formalin treatment (Figure 1).

ELISA mean absorbance values (Table 1) and IHA geometric mean antibody titers (Table 2) greatly increased after boostering in all groups of vaccinated chickens for both types of P. multocida (A and D). Overall ELISA mean absorbance values (Tabel 1) and IHA geometric mean antibody titers (Table 2) in sera of group I were higher than those of group II that were higher than those of group III. Similarly, the protection percent against the challenge exposure to virulent *P. multocida* serotypes A and D (Table 3) was better in group I than in groups II and III. The novel lysate EMG adjuvanted vaccine (group IV) scored higher ELISA mean absorbance values and better protection percent against virulent challenge exposure for both serotypes A and D of *P. multocida* than all other groups of vaccinated chickens (Tables 1 and 3).

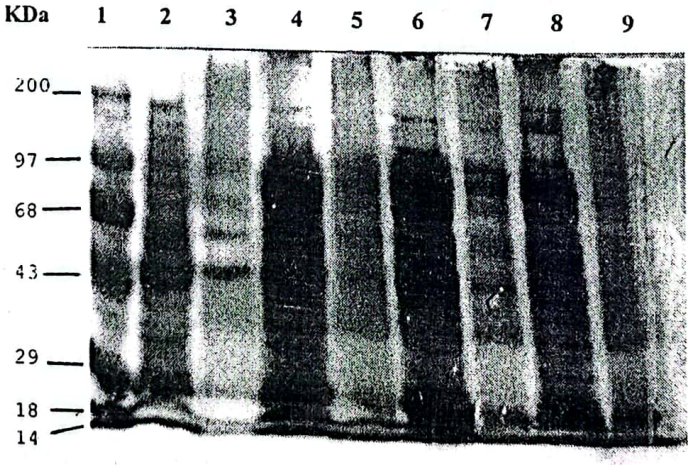

Figure 1 : SDS-PAGE analysis of antigenic composite of bacterial cell lysates (BCL) recovered from the 4 vaccinal serotypes of *P. multocida*, separated through 10% denaturing polyacrylamide gel and stained with 0.25% Coomassie Brilliant Blue R250 solution. Lanes: (1) Molecular mass protein standard 14-200 kilodaltons, kDa; (2, 4, 6 and 8) non formalin-treated BCL of *P. multocida* serotypes D:2, A:5, 9 and 8, respectively; (3, 5, 7 and 9) BCL of *P. multocida* serotypes D:2, A:5, 9 and 8, respectively, after formalin treatment.

Table (1) : ELISA mean absorbance values in sera of chicken vaccinated with local and reformulated fowl cholera vaccines

Group of chicken	Antigen type	PrevaccTiters	Intervals Post Vaccination										Post challenge	
			Primary vaccination				Secondary vaccination							
			1 week	2 weeks	3 weeks	Over-all mean	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	Over-all mean	2 weeks	4 weeks
(I)	A	0.129	0.965	1.182	1.190	1.112	1.365	1.460	1.580	1.590	1.600	1.519	0.865	0.988
	D	0.149	0.891	0.740	0.799	0.810	0.902	1.062	1.062	1.077	1.095	1.039	0.645	0.801
(II)	A	0.138	0.878	1.170	1.190	1.079	1.257	1.379	1.375	1.460	1.447	1.383	0.757	0.987
	D	0.130	0.872	0.959	0.998	0.943	1.007	1.120	1.133	1.135	1.196	1.118	1.505	1.094
(III)	A	0.125	0.707	0.767	0.896	0.790	0.917	1.005	1.050	1.150	1.345	1.093	0.857	0.957
	D	0.134	0.562	0.657	0.670	0.629	0.747	0.990	0.990	1.030	1.060	0.963	0.542	0.755
(IV)	A	0.183	0.787	0.987	0.990	0.921	1.486	1.495	1.595	1.598	1.680	1.570	1.325	1.247
	D	0.166	0.835	0.889	0.878	0.867	1.295	1.380	1.395	1.485	1.496	1.410	1.225	1.167
(V)	A	0.160	0.166	0.174	0.143	0.159	0.143	0.266	0.151	0.129	0.132	0.164	-	-
	D	0.123	0.129	0.118	0.121	0.123	0.127	0.171	0.162	0.231	0.165	0.171	-	-

Group (I): Immunized with modified fowl cholera, EMG adjuvanted bacterin.

Group (II): Immunized with modified fowl cholera double adjuvanted (SPT80 and EMG) bacterin

Group (III): Immunized with local conventional fowl cholera, SPT80 adjuvanted bacterin.

Group (IV): Immunized with the novel fowl cholera lysate, EMG adjuvanted vaccine.

Group (V): Control non-vaccinated.

Table (2) : Indirect haemagglutinating (IHA) geometric mean antibody titers (GMT) in sera of chicken vaccinated with loca and reformulated fowl cholera vaccines

Group of chicken	Antigen type	Prevace Titers	Intervals Post Vaccination										Post challenge	
			Primary vaccination				Secondary vaccination							
			1 Week	2 weeks	3 weeks	Over-all mean	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	Over-all mean	2 weeks	4 weeks
(I)	A	26	98	121	266	161.6	320	610	755	788	905	675.6	788	844
	D	21	86	113	211	136.6	299	345	422	455	485	401.2	610	755
(II)	A	30	113	130	149	130.0	343	485	520	577	610	507.0	970	970
	D	28	106	121	130	119.0	320	422	422	485	520	433.8	844	905
(III)	A	26	86	106	113	101.6	259	266	343	453	485	361.2	368	422
	D	25	80	92	106	92.6	211	260	297	394	422	316.8	320	349
(IV)	A	23	92	113	190	131.7	320	343	610	755	905	586.6	797	686
	D	21	86	120	149	118.3	343	422	520	610	844	547.8	755	905
(V)	A	20	26	28	23	25.6	25	30	25	28	26	26.4	-	-
	D	20	21	26	21	22.6	26	28	21	23	23	23.8	-	-

Group (I): Immunized with modified fowl cholera, EMG adjuvanted bacterin.
Group (II): Immunized with modified fowl cholera double adjuvanted (SPT80 and EMG) bacterin.
Group (III): Immunized with local conventional fowl cholera, SPT80 adjuvanted bacterin.
Group (IV): Immunized with the novel fowl cholera lysate, EMG adjuvanted vaccine.
Group (V): Control non-vaccinated.

Table (3): Challenge exposure test results of chicken vaccinated with local and reformulated fowl cholera vaccines using virulent *P. multocida* serotypes A and D.

Groups of birds	No. of birds/ group	No. of dead/survived birds after challenge exposure to virulent serotypes of *P. multocida*		Protection %		Lesion Score
		Type A	Type D	Type A	Type D	
(I)	26	0/13	1/13	100%	92%	+
(II)	26	1/13	1/13	92%	92%	+
(III)	26	2/13	1/13	84%	92%	+
(IV)	26	0/13	0/13	100%	100%	-
(V)	26	13/13	13/13	0%	0%	+++

Group (I): Immunized with modified fowl cholera, EMG adjuvanted bacterin.
Group (II): Immunized with modified fowl cholera double adjuvanted (SPT80 and EMG) bacterin.
Group (III): Immunized with local conventional fowl cholera, SPT80 adjuvanted bacterin.
Group (IV): Immunized with the novel fowl cholera lysate, EMG adjuvanted vaccine.
Group (V): Control non-vaccinated.
+ : mild lesions of fowl cholera +++ : severe lesions of fowl cholera

DISCUSSION

Vaccination plays a main role in the control of fowl cholera that is manifested usually as an acute septicemia, often fatal, causing considerable economic losses in all types of poultry farms worldwide (Derieux, 1978; Rhoades and Rimler, 1991). Adjuvants have been used for many years to boost the immunogenic response of antigens (McClimon et al., 1994). EMG is a unique oil-in-water adjuvant prepared from pharmaceutical grade oil, comprising uniformly dispersed micron sized oil droplets that unlikely result in tissue reactions. All ingredients including emulsifying agents of EMG, is approved by FDA for use in food and by the USDA for use in veterinary vaccines.

In this study, the commercial oil-in-water (EMG) adjuvant was used in comparison to the currently used water-in-oil (SPT-80) adjuvant in formulation of the local fowl cholera bacterin by both serological and biological assays. In parallel, use of non-formalin treated lysates of *P. multocida* serotypes as a novel vaccinal antigen emulsified in EMG was evaluated.

SDS-PAGE analysis revealed a considerable similarity in the electrophoretic patterns of cell lysates recovered from *P. multocida* types A:5, 8, 9 and D:2, manifested by 14-16 protein subunits at the molecular mass range of about 14-190 kilodaltons (kDa). However, a significant reduction in number protein subunits was observed in cell lysates of their counterparts after formalin treatment (Figure 1). This might be attributed to that inactivation procedure could destroy some important epitopes of the vaccinal antigen composite with a result of less efficient immune response (Monaco, 1992).

Use of serological assays specially the enzyme linked immunosorbent assay (ELISA) has become a practical method for predicting the immunological response of poultry to various vaccination programs (Briggs and Skeels, 1984). Clearly, ELISA mean absorbance values and IHA geometric mean antibody titers greatly increased after boostering in all groups of vaccinated chickens for both types of *P. multocida*. These results coincide with that obtained by Avakian et al. (1985) who stated that the secondary immune response in birds receiving commercial bacterins was much stronger than the primary one. Overall ELISA mean absorbance values, IHA geometric mean antibody titers and the protection percent against challenge exposure to virulent *P. multocida* serotypes A and D of group1 were higher than those of group II that were higher than those of group III. The present results are in full agreement with Zaher et al. (1977) and Azzam (1988) who stated that the survival rate increased post vaccination with two doses of polyvalent vaccine. In addition, the double oil adjuvanted bacterin (group II) gave earlier onset of immunity than water-in-oil adjuvant bacterin alone (group III) and generally more effective up to 20 weeks post vaccination (Mittal et al., 1979). Both indirect ELISA and IHA proved to be reliable assays for detection and titration of anti- *P. multocida* antibody in sera of vaccinated chickens. Superiority of ELISA compared to IHA test may be explained on account of that ELISA detects not only agglutinating but also other subpopulations of immunoglobulin G. (Scott McVey et al., 1990). The novel lysate EMG adjuvanted vaccine (group IV) scored higher ELISA mean absorbance values and better protection percent against virulent challenge exposure for both serotypes A and D of *P. multocida* than all other groups of vaccinated chickens. This could be attributed to use of EMG adjuvant or to that bacterial cell lysate might contain a composite of antigens: surface proteins, capsular polysaccharide and lipopolysaccharide in addition to other antigens such as fimbirial proteins (Confer et al., 1990). Also, This is in accordance with Delpy and MicChamshy (1949), Brogden and Rimler (1982) who stated that vaccination with *P. multocida* lysate had a good immunizing action. Also, use of bacterial culture including the culture medium might cause definite dilution of the bacterial antigen composite per final dose as in

groups I, II and III. However, the overall geometric mean antibody titres for IHA in group IV were less than group I. The ability of ELISA to detect IgG is far superior to the various agglutination tests (Marshall et al., 1981). Moreover, the anti- chickens IgG conjugate detects mainly IgG whereas IHA can measure any agglutinating antibody present particularly IgM. However, IgG has the major role in immune response against *P. multocida* (Marshall et al., 1981). Bacterial lysis of *P. multocida* after thawing from frozen status made studies on the immunogenicity of mildly disrupted bacterial preparation possible (Brogden and Rimler, 1982). Group (IV) gave higher protection percent due to the fact that *P. multocida* lysates can induce protection against both homologous and heterologous serotypes (Rimler et al., 1979). Nevertheless, serologic characteristics of *P. multocida* are based on the antigenic properties of the capsular and cell wall components (Manning, 1982). Thus, the immune response is directed mainly against the bacterial cell components not to the whole culture as in the rest of vaccinated groups. The cross protection factors (CPF) that present in the freeze thaw lysates are impeded during filtration. Release of active CPF in a soluble form after complete lysis of *P. multocida* by freeze thaw and enzymatic treatment have been reported for vaccine preparation (Rimler and Rhoades, 1981). Better immune response and protection percent against virulent challenge exposure were observed in groups (I), (II) and (IV) in which EMG was used as adjuvant either with formalized fowl cholera whole culture (I) and (II), or with non-formalized bacterial cell lysates (IV). It could be explained as that EMG acts by forming a mobile depot of antigen which can target immune response effector cells. The depot effect with slow release improve the presentation of antigen and provides a significantly better immune response and effectiveness of the vaccine (Hennessen, 1965).

In conclusion, all vaccine formulations used in this study including the current local vaccine are found reliable for seroconversion and protection of vaccinated chickens, with variable effectiveness. Also, EMG as an oil-in-water adjuvant produced a stable, potent, less viscous emulsion and easy injectable vaccine. Use of *P. multocida* lysates as a novel vaccinal antigen and EMG as an oil-in-water adjuvant, provided an advantageous formulation of the fowl cholera vaccine that conferred a 100% protection of chickens against virulent challenge. Moreover, it became much more safer vaccine for chickens and use of their meat in foods since it contains pharmaceutical grade oil of EMG with no formalin residues.

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