Vet.Med.J.,Giza. Vol.55, No.2. (2007):365-375.

PRELIMINARY PREPARATION AND EVALUATION OF A LOCAL LYSATE VACCINE FOR PROTECTION OF CHICKEN AGAINST *E. COLI* INFECTION

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Received: 9.9.2006.

Accepted: 12. 9. 2006.

SUMMARY

Four serotypes of *E. coli* (O1, O2, O6 and O128) were used in preparation of a local polylysate oil vaccine that was evaluated in comparison with a licensed imported one for protection of chicken against *E. coli* infection. Five groups, each of 20 chickens were vaccinated with the local vaccine subcutaneously (S/C) and intramuscularly (I/M) using single or double vaccinal dose and boostered after 2 weeks. Vaccinated and control chickens were challenged 2 weeks post second vaccination with the four virulent *E. coli* strains. No deaths occurred in all vaccinated chickens except one death case in one group. Survivors were necropsied one week post challenge. No gross lesions were observed in all vaccinated groups compared to those of non-vaccinated one. Polylysate vaccine gave similar results to that of the imported vaccine. Anti-*E.coli* antibodies were measured by an indirect enzyme linked immunosorbent assay (ELISA) in sera of all vaccinated and challenged chickens utilizing the four *E. coli* vaccinal antigens as individuals and as a polylysate. Moreover, no genetic variation could be detected among the four serotypes after digestion of their chromosomal DNA with XbaI enzyme. In conclusion, the polylysate antigens are suitable candidates for vaccine preparation to protect chickens from colibacillosis.

INTRODUCTION

Avian pathogenic strains of *E. coli* cause a number of related diseases in poultry including respiratory infections (Vandemaele et al., 2002), cellulitis (Gross, 1994) and colisepticemia, one of them is colibacillosis which is economically important to poultry production worldwide, colibacillosis can lead to death of poultry, carca condemnation and cost of treatment resulting in millions of dollars lost each year.

Ammonia and adverse environmental factors also predispose poultry to colibacillosis (Siccardi, 1975). Until now, antibacterial agents have been widely administreted for the treatment and control of colibacillosis in poultry flocks (Dhillon and Jack, 1996), but prolonged use of them, resulted in resistant bacterial strains which hazards to human health. The costs associated with using them have led to increase the trials to have alternative methods for protecting flocks against *E. coli* infection. Killed, subunit and live vaccines all have been evaluated in trials to develop an effective vaccine against colibacillosis in poultry (Melamed et al., 1991). Avian pathogenic *E. coli* (APEC) isolates commonly belong to certain serogroups, O1, O2, O78 and to other restricted numbers. Several studies have been permitted a reliable evaluation of the pathogenicity of *E. coli* for poultry indicated virulence factors identified on APEC as adhesins such as F1 and P Fimbrae and curli, aerobactin, non-sequestering system, capsule K1, temperature-sensitive haemagglutinin (TSH), resistance to bactericidal effects of serum and cytotoxic effect (Dho-Moulin and Fairbrother, 1999). Vaccines containing killed or attenuated virulent bacteria protect against infection with the homologous strain but are less efficient against heterologous strains (Zigterman et al., 1993). Bacterin has been effective against homologous experimental challenge (Panigrahy et al., 1984). Pilus vaccines have protected chickens against challenge with the homologous *E. coli* strain (Gyimah et al., 1986). Hence, vaccination for colibacillosis is not widely practiced because of the large variety of serogroups involved in field outbreaks so development of a bivalent or polyvalent poultry vaccine directed against 2 or more strains of *E. coli* would be benefit to the public health and increase poultry production. It has been shown that lysis of bacteria with enzyme and detergent could release immunogenic action against Pasteurella multocida (Rimler and Rhoades, 1981) homologous and heterologous serotypes. The purpose of this work was to develop a lysate vaccine that would protect poultry against more than strain of *E. coli,* evaluation of the efficacy of this vaccine to immune chickens against challenge with *E. coli* strains and to prove the abilities of solublizing agents to release active immunity.

MATERIAL AND METHODS

Bacterial strains:

E. coli strains groups O1, O2, O6, O128 were kindly obtained from Serology Department, Animal Reproduction Research Institute, Giza, Egypt. The strains were confirmed by standard techniques (Koneman et al., 1997) briefly, each strain was cultured initially in *E. coli* broth (Oxoid) incubated at 37°C for 24 hours then streaked on EMB, agar and incubated at 37°C for 18 hours. Colonies were randomly picked from EMB agar plates and confirmed by biochemical tests on API20E strips (Biomeriux) transferred on nutrient agar slopes then subjected to serological typing by slide agglutination using standard and monovalent *E. coli* antisera (Edward and Ewing, 1972).

Preparation of chromosomal DNA:

1.5 ml of bacterial culture of each strain were pelleted, resuspended in TE, lysed by SDS 10% and proteinase-K (Serva) cetyl-trimethyl ammonia bromide-NaCl (CTAB-NaCl). Purified by chloroform-isoamyl, phenol chloroform isoamyl and precipitated with 2 volumes absolute ethanol following a wash in 70% ethanol and DNA was resuspended in 50 μl sterile distilled water (Ausubel et al., 1992). Chromosomal DNA from each strain of *E. coli* were a digested with XbaI (Roche Diagnostics Penrberg, Germany) according to the manufacturer's instructions. The resulted DNA fragments after restriction endonuclease (XbaI) digestion were seperated by 0.7% agarose gel electropharesis.

Vaccines:

1. **Oil imported *E. coli* vaccine:**

   An imported inactivated subunit vaccine was supplied (Intervet, the Netherland) by Central Laboratory for evaluation of Veterinary Biologics, Abbasia, Cairo.

2. **Preparation of *E. coli* oil poly lysate vaccine:**

   E. coli strains were streaked on MacConkey agar plate and incubated overnight at 37°C. The bacterial growth of each strain were added to 100 ml of tryptic soy broth TSB (Oxoid) and brain heart infusion BHI (Oxoid), then incubated at 37°C for 16 hours with shaking. Cells were harvested by centrifugation at 6000 rpm at 4°C for 30 minutes, washed twice with normal saline and resuspended in lysis buffer, protein of these lysate antigens was measured at 546 nm by the spectrum diagnosis kit according to manufacturer instructions of spectrum diagnosis kit.

This lysate was emulsified in oil adjuvant consists of Whiterex 309 oil (9 parts), span 80 (one part), which represent oil phase. Tween 80 was added to lysate preparation in percentage of 2% (aqueous phase). The ratio of aqueous phase to oil phase was 1:2. All these substances were mixed together and belonged well in an emulsifier (Kalish, Montréal, Toronto, Canada, Model 9020 HP 1.25) until obtaining a stable emulsion.

After complete preparation, lysate vaccines were monitored for sterility and safety tests according to British Pharmacopoeia (2005).

Chickens:

One hundred chickens of two weeks of age were checked serologically for absence of *E. coli* antibodies and raised with sterile feed and water in battery cages.Throughout the Experimental period.

Vaccination of chickens:

Chickens were grouped into 5 groups 20 chickens each and treated as in the following table (I).

After 7 days, dead birds and survivors of post challenge were examined euthantized and necropised. Samples were taken for bacteriological examination and data obtained from all groups were used to evaluate the effects of vaccination according routine protocols for *E. coli* vaccine in Central Laboratory for evaluation of Veterinary Biologics, Abbasia, Cairo.

Indirect enzyme linked immunosorbent assay (ELISA):

ELISA was performed essentially as described by Coligan et al. (1994).

The antigens were diluted to a concentration of 1.5 µg/ml.ELISA 96 well immunoplates were coated with 100µl/ well of the lysate antigens of O1, O2, O6, O128 separately and mixed together, incubated at 4°C overenight. Serum was diluted 1:10 and added as 100µl/well in duplicates for each sample. Each microtitre plate contained positive and negative sera as well as a blank as controls.

The rabbit anti-chicken horseradish peroxidase conjugated antibody was diluted as (1:10, 000) and 100µl/well was added to each well.

The cut-off mean absorbance value for O1, O2, O6 and O128 were 1.23, 1.39, 1.43 and 1.35, respectively and 1.47 for polylysate antigen.

The above these cut-off values a serum samples was regarded as positive.

Table (I): Vaccination schedule of chicken groups

Vaccinated groups	Type of vaccine	Route and dose of vaccine	Bleeding	Challenge
Group (A)	Lysate vaccine	0.5 ml S/C	All chickens were bled 1, 2 weeks post first vaccination and 1, 2, 3, 4 weeks post second vaccination. Sera were separated and examined serologically by ELISA	Each chicken in each group was challenged with 0.1 ml S/C of 24 hours *E. coli* old culture (1×10^8 CFU) (mixed of the 4 *E. coli* strains O1, O2, O6 and O128)
Group (B)	Lysate vaccine	0.5 ml I/M		
Group (C)	Imported vaccine	0.5 ml S/C		
Group (D)	Lysate vaccine	1.0 ml S/C		
Group (E)	Unvaccinated control	-		

Each chicken in each group received two doses of vaccine two weeks apart.

RESULTS AND DISCUSSION

Escherichia coli infections are being increasingly detected among poultry flocks, indicating the growing importance of this pathogen to industry. The objective of this work was to determine the immune response of chickens to an experimental respiratory tract infection with APEC so as to identify vaccine candidate immunogens. Well characterized *E. coli* serogroups that were most frequently isolated from septicemic chickens in Egypt and evaluated the chicken immune response to lysate antigens of these strains, that are potentially suitable as vaccines. Lysate protein concentration was measured separately and collectively. Each strain contained protein concentration less than that found in mixture except O2 (2.5 mg/ml), mixture 1.59 mg/ml, O1, O6 and O128 were 1.38, 1.27 and 1.23 mg/ml. Lysate vaccines have all the components of bacterial cells and were adjusted in 0.5 ml dose containing 1.0 mg/ml of total protein. This study demonstrated that vaccinated chickens with the lysate vaccine induced protection similar to that obtained with imported vaccines against colibacillosis, when vaccinated chickens were challenged with the virulent *E. coli* strains. Choosing chickens at 2 weeks of age for vaccination was due to previous studies indicated that vaccination before 14 days of age is ineffective (Trampel and Griffth, 1997) due to maternal immunity and immaturity of immune system of young chicken, in addition to older chickens are more resistant to experimental infection with *E. coli* (Frommer et al., 1994).

Mineral oil emulsions have been used to potentiate the antibody response and prolong the action of the antigens (Cox and Coulter, 1997). Tween 80 is known to cause a reduction in both viscosity and interfacial tension and increase the coagulation rate of the aqueous globules at water in oil emulsion (Chiejina and Sewell, 1974).

In this experiment, birds were vaccinated twice with the local lysate vaccine with different route and the imported vaccine S/C only, these birds were challenged, necropised 7 days later and scored for the lesions. Tissue samples were also taken to reisolate the challenge strain from all birds. Mortality, macroscopic lesion scores are shown in Table (6). Lesion scores for chicken in groups A and B vaccinated with lysate vaccine and challenged were similar to that injected with imported vaccines group (C) regardless the route of vaccination and bird to bird variation, and were less than those of group E, unvaccinated.

No great remarkable difference observed concerning the route of administration, one chicken from group (B) (Table, 6) showed macroscopic lesion and 1 dead chicken than groups A and C No mortality or gross pathologic changes occurred in group D that received double dose of lysate vaccine.

Using of combination of serogroup and DN digestion information in order to assess the heterogeneity of *E. coli* associated with avian colibacillosis in chickens. 3 of the 4 isolates (O1, O2, O6, O128) were digested. One of the isolate did not yield bands after repeated attempts. On the basis of results of other studies, this condition is likely due to the degradation of DNA during digestion by endogenous endonucleases (Izumiya et al., 1997) and no difference could be observed on the gel with other strains.

The antibodies response in sera as shown in (Tables, 1, 2, 3 and 4) to the single and multiple antigens were similar to that of imported vaccines. The antibody responses to all the antigens in serum were always significantly higher in the vaccinated, challenged group (A, B, C) than in the unvaccinated challenged group (E). *E. coli* antigens were attributed to serum antibody by ELISA gave moderate titre of serum (positive antibodies) at 1st week, high titre 3rd and 4th week and higher titre post challenge, indicating further maturation of humoral immune responses generated by lysate vaccine. imported vaccine produced a little raise in humoral antibody response to *E. coli* than lysate vaccine this may be due to it has been developed on the basis of F1 and FT antigen only.

Escherichia coli bacterins (Deb and Harry, 1978) subunit vaccines (Gyimah and Panigrahy, 1985) and live vaccines (Formmer et al., 1994) all in duced good protection against colibacillosis bu an effective vaccine for APEC induced diseas should protect against all disease associated sero groups. The four antigens tested induced stron antibody response post infection in serum an were nearly similar. A combination of O1, O2 O6, O128 is seem to cover good range of most o virulent factors of APEC and the whole lysat vaccine contain soluble and insoluble component has been shown to be immunogenic in turkey (Brogden and Rimler, 1983) elicited a protectiv immune response against virulent *E. coli* chal lenge, in this experiment.

Overall, a strong correlation was found betwee antibody response and low lesion score indicatin good protection. As a conclusion, these studie demonstrated that poly-antigen lysate are possibl candidates for a vaccine against *E. coli* infectio in chickens. However, the level of protection wa like that of the commercial imported vaccine an it is desirable to increase, it is easier in prepara tion, safe time, inexpensive, does not need selec tive media. It has shown to be safe, immunogeni and effective against heterologous *E. coli*, whe the laboratory studies indicate protection agains experimental challenge, a field trial is needed t test the vaccine in natural environmental condi tion.

Table (1): ELISA mean absorbance value of chicken sera using the *E. coli* serotype "O1" antigen

	Chick group	WPV 1st dose		WPV 2nd dose		W.P.Ch.	
		1st week	2nd week	1st week	2nd week	3rd week	4th week
Vaccinated groups	Group (A)	1.9	2.04	2.1	2.9	3.71	3.9
	Group (B)	1.8	2.0	2.1	2.8	3.5	3.8
	Group (C)	1.9	2.1	2.2	2.5	3.71	3.9
Non-vaccinated	Group (E) Control	0.65	0.65	0.65	0.65	0.65	0.65

W.P.Ch.: Weeks Post Challenge
WPV: Weeks Post Vaccination

Table (2): ELISA mean absorbance value of chicken sera using the *E. coli* serotype "O2" lysate

	Chick group	WPV 1st dose		WPV 2nd dose		W.P.Ch.	
		1st week	2nd week	1st week	2nd week	3rd week	4th week
Vaccinated groups	Group (A)	1.85	1.9	2.1	2.3	3.3	3.4
	Group (B)	1.70	1.8	2.9	2.5	3.0	3.1
	Group (C)	1.9	2.2	2.5	2.7	3.2	3.9
Non-vaccinated	Group (E) Control	0.65	0.65	0.65	0.65	0.65	0.65

W.P.Ch.: Weeks Post Challenge
WPV: Weeks Post Vaccination

Table (3): ELISA mean absorbance value of chicken sera using the *E. coli* serotype "O6" antigen

	Chick group	WPV 1st dose		WPV 2nd dose		W.P.Ch.	
		1st week	2nd week	1st week	2nd week	3rd week	4th week
Vaccinated groups	Group (A)	1.9	2.2	2.5	2.8	3.1	3.4
	Group (B)	1.8	2.1	2.3	2.5	2.8	3.2
	Group (C)	1.9	2.1	2.4	2.9	3.2	3.9
Non-vaccinated	Group (E) Control	0.65	0.65	0.65	0.65	0.65	0.65

W.P.Ch.: Weeks Post Challenge
WPV: Weeks Post Vaccination

Table (4): ELISA mean absorbance value of chicken sera using the *E. coli* serotype "O128" antigen

	Chick group	WPV 1st dose		WPV 2nd dose		W.P.Ch.	
		1st week	2nd week	1st week	2nd week	3rd week	4th week
Vaccinated groups	Group (A)	1.9	2.1	2.4	2.6	2.9	3.3
	Group (B)	1.8	1.9	2.2	2.5	2.8	3.0
	Group (C)	1.9	2.2	2.3	2.7	2.9	3.9
Non-vaccinated	Group (E) Control	0.65	0.65	0.65	0.65	0.65	0.65

W.P.Ch.: Weeks Post Challenge
WPV: Weeks Post Vaccination

Table (5): ELISA mean absorbance value of chicken sera using the *E. coli* mixed lysate antigen

	Chick group	WPV 1st dose		WPV 2nd dose		W.P.Ch.	
		1st week	2nd week	1st week	2nd week	3rd week	4th week
Vaccinated groups	Group (A)	2.1	2.1	2.3	2.9	3.1	3.6
	Group (B)	1.9	2.1	2.1	2.3	2.9	3.0
	Group (C)	2.1	2.2	2.5	2.7	3.2	3.9
Non-vaccinated	Group (E) Control	0.65	0.65	0.65	0.65	0.65	0.65

W.P.Ch.: Weeks Post Challenge
WPV: Weeks Post Vaccination

Table (6): Lesion scores of chickens vaccinated with lysate vaccine imported vaccine and non-vaccinated after challenge chicken

Chick group	No. of birds with each lesion						Percentage
	0	1	2	3	4	5	
Group (A)	14	6	-	-	-	-	70 %
Group (B)	12	6	1	-	-	1	60 %
Group (C)	15	5	-	-	-	-	75 %
Group (E)	-	-	2	10	6	2	0 %

5: Dead birds
4: Severe and extensive fibrincus air sacculitis and pericarditis or hepatitis
3: Bilateral air sacculitis, pericarditis or hepatitis
2: Moderate air sacculitis, pericarditis or hepatitis
1: Cloudy air sacculitis, pericarditis or hepatitis
0: No lesions

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