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PREPARATION AND EVALUATION OF THE PROTECTIVE EFFICACY OF *PSEUDOMONAS AERUGINOSA* IRRADIATED VACCINE IN CHICKENS

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SUMMARY

A total of 75 *P. aeruginosa* isolates were secured out of 280 samples from diseased chickens suffering from different respiratory manifestations. The isolates were characterized and serotyped. The most prevalent serotypes were O3 and O4. An inactivated bivalent *P. aeruginosa* vaccine was prepared from these serotypes. The inactivation was achieved through irradiation with Cobalt 60. The quality control analysis of the prepared irradiated vaccine indicated that it was safe and its use was not associated with any clinical distress. Application of ELISA for evaluation of humoral immune response developed against *P. aeruginosa* in the vaccinated chicken groups showed significant increase in antibody titers, which was influenced by the route of immunization. The protection rates post challenge reached 100%, 96% and 90% among the intramuscularly, subcutaneously and orally vaccinated chicken groups, respectively. Also, vaccination of egg laying hens with the prepared vaccine stimulated formation and concentration of *P. aeruginosa*-specific IgY in the egg yolk.

INTRODUCTION

Pseudomonas aeruginosa is an environmentally ubiquitous Gram-negative bacteria that causes severe infections among immuno-compromised hosts (Cripps et al., 1997). This bacterial species is an opportunistic pathogen that can affect human, animals and birds.

In birds infections with *P. aeruginosa* is associated with respiratory manifestations, diarrhoea and septicaemia with high morbidity and mortality

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rates, particularly in flocks under massive antibiotic therapy (Abdalla, 1987 and El-Ged et al., 1993). In young chicks *P. aeruginosa* produces progressive weakness, dyspenia, considerable oedema of the head and cheesy deposits on the serous surfaces lining the air sacs and peritoneal cavity. Also enteritis, congestion of internal organs and petechial hemorrhages on the coronary fats have been reported (Awaad et al., 1981; Andreev et al., 1982, Gross, 1984, Kheir-Eldin et al., 1986 and Riad, 1994). Nevertheless, *P. aeruginosa* is incriminated to be one of the important causes inducing marked reduction in egg hatchability and death of the chick embryos (Utomo and Poernomo, 1990).

P. aeruginosa is naturally resistant to most antibiotics and quickly develops resistance to the commonly used ones (Nakae et al., 1997). Also it is resistant to many disinfectants including quaternary ammonium compounds, dettol and cetrimide that are included in some commercial media used for selective isolation of *P. aeruginosa.*

Inspite of the important health and economical problems that can affect poultry industry through *P. aeruginosa*, no vaccine or vaccination trials in chickens could be traced in the available literature. Therefore, the aim of the present investigation was to prepare a safe effective vaccine from the most prevalent *P. aeruginosa* serotypes and to evaluate its protective potential in chickens. Also, to study the production of *P. aeruginosa*-specific IgY antibodies in the egg-yolk of immunized egg laying hens as an approach to control the still-in death embryos problem caused by *P. aeruginosa.*

MATERIAL AND METHODS

1- Isolation of *P. aeruginosa* from chickens:

- Samples: A total of 240 sinus swabs were collected from 4-8 weeks diseased chickens suffering from respiratory manifestations at the Poultry Farm, Faculty of Agriculture, Cairo University. In addition to 40 samples that were collected separately from heart blood (12), sinuses (16), and lungs (12) of freshly dead chickens. The collected swabs were inoculated into nutrient broth and incubated for 24 hr at 37°C. An inoculum was cultivated onto Pseudosel agar, nutrient agar, and MacConkey's bile salt agar slants and incubated at 37°C for 24-48 hr and examined.
- Identification of the isolates: The suspected colonies were purified and identified according to Quinn et al. (1994). Serotyping of the isolates was performed with slide agglutination test using *P. aeruginosa*- specific antisera (Difco, Detroit, Michigan, USA).

2- Preparation of bivalent irradiated *P. aeruginosa* Vaccine:

Preparation of dense culture of *P. aeruginosa* serotypes O3 and O4: The two serotypes were inoculated into nutrient broth separately and incubated at 37°C on rotatory shakers for 18

hr. The cells were then harvested by centrifugation at 1500 xg for 15 min, washed three times with phosphate buffered saline (PBS) pH 7.4 and resuspended in equal volume of PBS. The Two serotypes were lyophilized according to Rybinkar et al. (1989). Thereafter, the number of colony forming units (CFU)/mg of the lyophilized powder was determined for each serotype (Gebhardt, 1996).

Preparation of *P. aeruginosa* vaccine: The lyophilized powder of the two serotypes O3 and O4 were inactivated using 5 Kilo Gray (KGy) irradiation dose from a Cobalt source at room temperature. The calculated exposure dose rate was 1.48 KGy/hr for 205 min. This dose rate was determined in a preliminary study to induce complete inactivation of *P. aeruginosa*. The two inactivated serotypes were mixed in amounts representing equal CFU for each serotype and used for the immunization experiments.

Quality control analysis of the prepared vaccine: The prepared bivalent *P. aeruginosa* vaccine was tested for inactivation, purity, sterility and safety according to the Standard international protocols described by British Veterinary Codex (1970) and Code of the American Federal Regulation (1985).

3. Experiment I: Evaluation of the immunizing potential and protective efficacy of the prepared vaccine in broiler chicks: A total of 1320 one day old chicks obtained from a commercial source were housed under hygienic conditions and provided with food and water adlibitum. The chicks were divided into five groups (264 chicks/group). Group (A) was the unvaccinated control group. Groups (B) and (C) were vaccinated intramuscularly and subcutaneously, respectively, with 1 mg of the bivalent irradiated *P. aeruginosa* vaccine containing $1X10^9$ CFU/mg ($5X10^8$ CFU from each serotype) suspended in 0.5ml sterile PBS. Vaccination was carried out three times at 2 weeks intervals. Chicks in group (D) were orally vaccinated with a dose of 0.5 mg containing $5X10^8$ CFU/chick, then vaccination was repeated orally using the same dose for 6 times at one-week interval. Group E was injected I/M at three days of age with 0.5 ml of the prepared vaccine (containing $1X10^9$CFU). This was followed by two doses given orally at 2 weeks intervals (0.5 mg/chick suspended in PBS).

3.1. Detection of *P. aeruginosa* -specific antibodies in serum samples from the immunized chicks:

- Blood samples: Non heparinized blood samples were collected before vaccination and every two weeks post vaccination up to 6 weeks. The collected samples were allowed to clot overnight at 4°C, then centrifuged at 1500 xg for 10 min. The separated serum samples were stored at -20°C till evaluated.

- ELISA procedure: The test was performed according to the method described by Leitner et al. (1990). 96 wells ELISA microtiter plates were coated with sonicated *P. aeruginosa* antigen (20ug/well) dissolved in carbonate bicarbonate buffer at pH 9.6. The plates were then blocked with 1% bovine serum albumin. These plates were used for titration of the collected serum samples for *P. aeruginosa*-specific antibodies. Double-fold serial dilution from 1/10 to 1/5120 was made from the tested serum samples. The serum dilutions were dispensed in the microtiter plates (50 ul/well) and the plates were incubated at 37°C for 60 min. The plates were washed 3 times using PBS-containing 0.05% Tween20 (PBS-T). Horseradish peroxides anti-chicken immunoglobulin (Dilution 1 / 10000), purchased from Sigma (Art No. 9046) USA, was dispensed in the wells of microtiter plates (50ul/well). The plates were incubated at 37°C for 30 minutes, then washed 3 times using PBS-T. To all wells in the used plates o-phenylene diamine (OPD) substrate was added (50ul/well), incubated at 37°C for 15 min, then the reaction was stopped by addition of 25 ul/well of 1.25 M sulfuric acid. The plates were read using ELISA reader at 405 nm. Interpretation of the obtained results was analyzed according to Gast et al. (1997).

3.2. Challenge test: Two weeks after the last immunization dose, the chickens were challenged by subcutaneous injection of 1 ml of 16 hr nutrient broth culture containing $6X10^8$ CFU/ml of *P. aeruginosa* serotype O3. Following challenge, all chickens were kept under daily observation for 2 weeks. The morbidity rate, lesion scores and mortality rates were recorded. All dead chickens and some of the survivors were subjected to postmortem inspection. The organs were cultured onto nutrient agar for reisolation of *P. aeruginosa.*

4. Experiment 2: Vaccination of laying hens for production of *P. aeruginosa*-specific IgY yolk antibodies: A total of ten white egg laying Leghorn hens (6-months old) were immunized I/M with 1 mg ($1X10^9$ CFU)/hen of the prepared bivalent *P. aeruginosa* vaccine. Vaccination was repeated 3 times at two weeks intervals.

4.1. Detection of *P. aeruginosa*-specific IgY antibodies in the egg yolk of immunized hens:

- Egg collection: Eggs from laying hens were collected just before immunization and every two weeks after each immunization dose up to 6 weeks. The collected eggs were kept in refrigerator until examined and used for separation of the IgY.
- Extraction of IgY antibodies from the egg yolk of immunized hens (Denzin, 1998): After careful separation of the egg yolk from the egg white, the yolk was rolled on filter paper in order to free it completely from

albumin material. The IgY was extracted from the egg yolk using ammonium sulphate precipitation method (Hudson and Hay, 1980). The prepared IgY was filtered sterilized using 0.45 um filter and its protein concentration was determined by measurement at 280 nm. According to Hudson and Hay (1980) a concentration of 0.69 mg chicken IgY/ml gives at 280 nm an optical density (OD) equal to 1. This data was used in calculation of IgY concentration in the prepared egg yolk. The IgY preparations were stored at -20°C till analyzed.

- Application of ELISA for determination of the concentration of *P. aeruginosa* specific antibodies in the IgY preparations: It was carried out as mentioned before using egg yolk IgY instead of serum

RESULTS AND DISCUSSION

Results shown in table (1) revealed that the incidence of *P. aeruginosa* among the examined chickens was 26.79%. On the other hand the prevalence rate reached 31.25% and 26.25% in swabs collected from sinuses of freshly dead chickens and diseased living chickens showing respiratory manifestations, respectively. Chakrabarty et al. (1980) isolated *P. aeruginosa* from chickens suffering respiratory illness. Also Abdalla (1987) isolated *P. aeruginosa* from sinuses, trachea, lungs and air sacs in chicken flock with respiratory affections. Furthermore, Lin et al. (1993) recovered Pseudomonas species from respiratory tract of birds with long history of respiratory illness and from bone marrow of dead birds.

Table (1): Rate of isolation of *P. aeruginosa* from diseased and freshly dead chickens

Source of isolates	Number of examined samples	Positive samples	
		Number	%
Diseased chicks:			
Sinuses	240	63	26.25
2-Freshly dead chicks:			
Sinuses	16	5	31.25
Heart blood	12	3	25.00
Lungs	12	4	33.33
Total		75	26.79

Table (2): Serotyping of *P. aeruginosa* isolates recovered from the examined chicks.

Serotypes	*P. aeruginosa* isolates recovered from:		Total number of isolates	%
	Diseased chickens	Dead chickens		
O3	32	6	38	50.7
O4	27	5	32	42.7
Untypable	4	1	5	6.6
Total	63	12	75	100

* The percentage was calculated to the total number of examined samples.

In the present investigation, *P. aeruginosa* isolates were recovered from heart blood of freshly dead chicks with an incidence rate of 25.00%. This observation seems to indicate that *P. aeruginosa* might invade the respiratory tracts and cause septicemia. These results coincide with McCabe (1986) who recorded that *P. aeruginosa* was the most frequent Gram negative bacteria that gain access to the blood from extravascular septic foci via lymphatic or by the direct invasion of small blood vessels within a local site of infection. Tancrede and Andremont (1985) reported also that the gastrointestinal tract was the primary reservoir of opportunistic bacteria and more than 80% of patients with *P. aeruginosa* bacteraemia were intestinal carriers of the same strain. Previous reports have indicated that gut bacteria can cross the gastrointestinal mucosal barrier and spread systematically, a process called bacterial translocation (Berg and Carlington, 1980 and Deitch et al., 1986).

All *P. aeruginosa* isolates were serotyped according to the scheme of Verder and Evans (1961). Table (2) shows that the predominant serotype was O3 (50.70%), followed by serotype O4 (42.7%). Schalm et al. (1967) and Srinivasan (1977) recorded that *P. aeruginosa* O3 and O6 were usually identified from apparently healthy and diseased chickens. Also, Schildger et al. (1989) isolated *P. aeruginosa* O3 from diseased chickens with an incidence rate of 13%.

The quality control analysis of the prepared vaccine proved its safety where no adverse reactions or abnormalities were observed in the immunized chickens during the whole experimentation period.

As shown in table (3) the immunogenicity of the prepared vaccine was evaluated by detection of *P. aeruginosa* -specific antibodies in sera of vaccinated chickens using ELISA. It appears clear that

Table (3): Geometric Mean antibody titers (GMT) against *P. aeruginosa*-measured with ELISA in sera from the unvaccinated and differently vaccinated chicken groups.

Time of serum Collection	GMT in sera from chicken groups differently vaccinated with *P. aeruginosa* vaccine				
	A	B	C	D	E
0 weeks	-	-	-	-	-
2 Weeks	-	640	640	320	2560
4 weeks	-	1280	5120	2560	5120
6 weeks	-	5120	5120	5120	5120

Group A: Unvaccinated chicks. Group B: Intramuscularly vaccinated chicks.
Group C: Subcutaneously vaccinated chicks. Group D: Orally vaccinated chicks.
Group E: Intramuscular followed by oral vaccination

the route of immunization influenced the intensity of the developed immune response. The Geometric mean titers (GMT) of antibodies increased from a zero pre-vaccination level to 640 at 2 weeks after primary immunization both among the subcutaneously and intramuscularly immunized chicken groups. After 2 weeks post boostering, the GMT reached 5120 and 1280, respectively. Meanwhile, the GMT reached to 320 and 2560 then increased to 2560 and 5120 after the first and second doses of vaccination among the orally immunized group and group E, which was immunized I/M in addition to oral administration. These results coincide with Jang et al. (1999) who reported higher antibody titers against *P. aeruginosa* following I/M immunization in humans. Pier and Thomas (1983) indicated that a single dose of *P. aeruginosa* bacterin was sufficient for the maintenance of elevated levels of specific antibody over an extended period of time. Meanwhile, additional boostering dose through the intratracheal route increased the immune response and enhanced the bacterial clearance as recorded by Thomas et al. (2001). This data comes approximately in agreement with the results obtained in the present work, where the antibody GMT reached to 5120 after 2 weeks from the third vaccinal dose.

At the present time, little is known about the antibody level required to confer protection against *P. aeruginosa* in chickens. In the present study, however, higher antibody levels were associated with greater rates of protection. It would appear, therefore, that antibody levels provide a marker for protection and are essential for protective immunity.

In conclusion it could be demonstrated that the irradiated vaccine against *P. aeruginosa* is safe and highly immunogenic in chickens and all routes induced successful immunization, but the I/M route was relatively more better than both S/C and oral ones.

The present work clearly confirmed the protective efficacy of the prepared vaccine. Significant protection rates were achieved in the differently immunized chicken groups following challenge with fresh culture of *P. aeruginosa* as compared with the unvaccinated control group (Fig.1). A protection rate of 100% and 96% was recorded in chicken groups vaccinated I/M and S/C, respectively. In this concern, Gilleland et al. (1980) and Sordelli et al. (1983) concluded that systemic immunization in rat model with purified *P. aeruginosa* proteins protect against acute respiratory infections caused by this bacterial species.

Freihorst et al. (1989) reported that oral immunization with *P. aeruginosa* could not induce the same protective potential as systemic immunization in a rat model. This fact agreed with our results, where in the orally vaccinated chicken group a protection rate of 90% was recorded. However, such protection rate although still lower than those approved by the other routes (I/M and S/C), it remains to be significantly higher protection rate that support its application on flocks.

Vaccinated chickens were protected against active respiratory infections in that they gained body weight, suffered low morbidity and mortality and the lesions developed post challenge were very mild when compared with the unvaccinated control group. Chickens in this group suffered severe respiratory manifestations, conjunctivitis, swelling of the hock joints and subcutaneous oedema.

In the second experiment an attempt was made to detect *P. aeruginosa*-specific antibodies (IgY,) in the eggs of vaccinated hens. Results of ELISA titers of IgY in the extracted egg yolk samples as shown in table (4) revealed a GMT of 640 at two weeks after primary vaccination. The GMT increased to 5120 after boostering as well as the total protein content was significantly increased (Table, 5). These results indicate that vaccination of hens with the irradiated *P. aeruginosa* vaccine induced significant concentration of *P. aeruginosa*-specific IgY antibodies in the eggs with subsequent induction of good protective effect on the embryos and preventing *P. aeruginosa* -induced reduction of egg hatchability and still-in-death embryos.

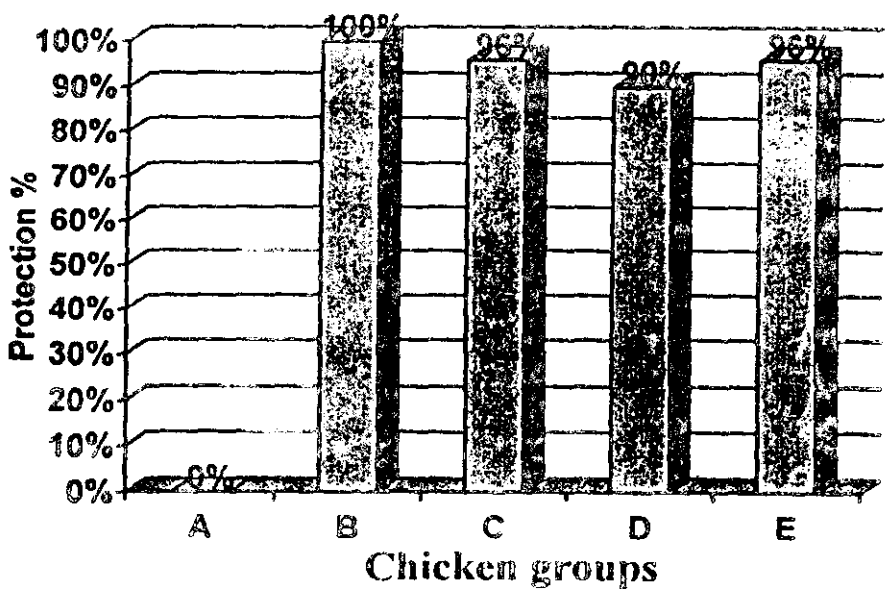
Table (4): Mean GMT of *P. aeruginosa*-specific antibodies measured with ELISA in egg yolk of vaccinated hens.

Time of egg collection	GMT of *P. aeruginosa* -specific antibodies									
	10	20	40	80	160	320	640	1280	2560	5120
0 weeks	-	-	-	-	-	-	-	-	-	-
2 Weeks	+	+	+	+	+	+	+	-	-	-
4 weeks	+	+	+	+	+	+	+	+	+	+
6 weeks	+	+	+	+	+	+	+	+	+	+

Table (5): The total protein content of egg yolk collected from hens vaccinated with the irradiated *P. aeruginosa* vaccine.

time of collection post vaccination	total protein content (gram %)
Pre-immunization	31.37
2 weeks post the first dose	44.10
2 weeks post the second dose	50.46
2 weeks post the third dose	53.72

Fig. (1): Influence of the route of immunization on the protection rate in chickens immunized with the irradiated bivalent *P. aeruginosa* vaccine.



Chicken groups
A- Unvaccinated gorup. B- Intramuscularly vaccinated group
C- Subcutaneously vaccinated gorup. D- Orally vaccinated gorup.
E- Chicks were immunized intramuscularly follwed by oral vaccination.

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