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Application of Single-Radial Immuno-diffusion test for evaluation of uropathogenic *Escherichia coli* sonicated immunogen in chick embryos

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Abstract
This study intended to evaluate the effect of whole cell sonicated uropathogenic *Escherichia coli* antigen (WCS. Ag) against the challenged of the infectious dose 50 (ID50) in chicken embryos. Twenty chicken embryos / one day age were divided into 2 equal groups. The first group was inoculated with 0.1 ml of WSC, Ag, the second group (control) was injected with 0.1 ml phosphate buffer saline (PBS). Blood samples were collected at 2 and 4weeks after inoculations. Single Radial Immuno-Diffusion assay (SRID) was used to test the significance of the effects of treatments. Fifty groups of eggs (10 eggs per group) were used to determine the infectious dose 50 (ID50). All chicken embryos of the treated and control groups were challenged with 3ID50 (2×10^6) of virulent uropathogenic *Escherichia coli*. The SRIT showed significant increase in antibody titers in the experimental group compared with the control group. The post challenge with ID50 of *E. coli* revealed that all chicken embryos in control group suffered from severe clinical signs of colibacillosis. In conclusion, this study indicated the ability of WSC. Ag to protect chick embryo against the challenged of the infectious dose 50 (ID50).

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Introduction

Escherichia coli and other gram-negative bacilli, can cause non–food-related illnesses, including blood infections (septicemia), inflammation of the brain and spinal cord (meningitis) in newborn children, in addition to urinary tract infections in children and adults.(American Academy of Pediatrics, 2007). *E coli* is a zoonotic enteric pathogen that cause human gastrointestinal illnesses (Amezquita *et al*., 2014). *Escherichia coli* is a part of the normal microflora in the poultry intestine, but certain strains, such as those

designated as avian pathogenic *E. coli* (APEC), spread into various internal organs and cause the systemic fatal disease colibacillosis (Barnes *et al.*, 2003; McPeake *et al*., 2005). Colibacillosis is one of the main causes of economic loss in the poultry industry worldwide. Losses attributable to colibacillosis in broilers have been described previously (Cavero *et al*., 2009). A distinct syndrome associated with colibacillosis was found in laying hens that is characterized by acute mortality without prior clinical signs of the disease and without a significant effect on egg production or quality. Necropsy examination showed that infected layers had lesions indicative of colisepticemia. The majority of colisepticemia outbreaks occur around the period of peak egg production (Vandekerchove *et al*., 2004, Trampel *et al*., 2007). Several serotypes (including O1, O2, and O78) caused colibacillosis and a range of virulence factors have been described previously (Blanco *et al*., 1998). The potential virulence factors, including adhesions, iron acquisition systems, hemolysin, antibactericidal factors, and toxins, have been implicated in promoting the severe disease situations in avian species (Janßen *et al*., 2001; Ewers *et al*., 2004, 2007). The fundamental reaction of immunology involves the interaction of antibodies (Ab) and antigens (Ag). These interactions are useful in the defense of the body against bacteria. The defense capabilities are depending upon the recognition of antigens by humoral components of the immune system. It is well known that specific antibodies are produced in response to exposure to the antigen and can be estimated by different immunological test such as SRID assay (http://faculty.mu.edu.sa/public/uploads/1336336004.4768Exercise_1.1 Radial_Immunodiffusion.pdf). Vaccines from different sources clearly differ by *in vitro* tests including single the radial immunodiffusion assay (SRD) but the significance of the differences, if any, is unknown (Nicoll *et.al*, 2013; Kelly *et.al*., 2015). This study was designed to evaluate the humoral immune response in chicken embryos following exposure to WSC antigen of uropathogenic *E. coli*.

Materials and Methods

Samples collection

Escherichia coli were isolated from the urine of the children. Urine samples were cultured on different selective media. Then, the bacterial isolates were identified by different biochemical tests (Quinn *et al.*, 2004). The bacterial isolates were confirmed at the microbiological laboratory in Alhashmia hospital, Ministry of Health/ Iraq and according to (Mitov *et al*., 1992).

Bacterial isolation

A bacterial suspension of *E. coli* was made from overnight agar culture and it was sonicated for 50 minutes intervals in a water cooled sonicator oscillator at 40 MHZ per second. Later on the homogenate was centrifuged three times by using a cooling centrifuge at 6000 RPM/ 30 minutes each time to remove cellular debris. The supernatants were passed through a 0.22 µm Millipore filter and stored at (-20^{o}C) until used. Protein content of the antigen was determined by biuret protein assay. Sterility and safety were done for the WSC antigen before using and according to OIE (2004).

Experimental study

Twelve healthy chicken embryos aged 1 day were used to evaluate the efficacy of the prepared antigen. All chicken embryos had negative fecal bacteriological culture for *E.coli*. They were reared in separate cages in the Animal House of Veterinary College, University of Alqasim. The animals were divided equally into two groups. The first group was Immunized with WSC Ag and inoculated twice at day one and after one week throw mouth at dose of 1 ml containing $2x10^6$ CFU/ml. The second group (control) was inoculated with 1 ml of PBS. Blood samples were collected from all groups at 2^{nd} and 4^{th} week post-inoculation and according to the method described by Johnson *et al.*, (2008). The humoral immunity was evaluated by SRID assay as described by Mancini *et al.*, (1965).

The infectious dose 50 (ID50) was estimated by using two fold dilution for counting bacteria by viable bacterial plate count method (Quinn *et al*., 2004). Forty healthy egg embryos (age 18 days) were divided into (4) groups (10 per group). Four groups of eggs embryos were injected (Figure. 1) in allantoic sac with 0.1 ml of calculated CFU diluents (tubes number 4, 5, 6, 7), and the other four group was considered as a control group and were injected with PBS. All groups were monitored for hatched day to calculate the total live and dead embryos, and also to estimate the ID50 according to (Reed and Muench, 1938). After 4th week, all immunized and control chicken embryos were challenged and inoculated with 3LD50 virulent *E. coli* to evaluate the efficacy of prepared the antigen in inducing immune response.

Statistical Analyses

All data were analyzed by using statistical package for social science (SPSS) version 13.0, the two-way analysis of variance was conducted to test the significance of effects of groups and periods in the post injection on the examined traits. The statistical differences among means of the different treatments were tested By Duncan's multiple range test.

Results

The results of SRID reactions zones (Figure 2) appeared as precipitation in immune-plates that containing antibody to the purified whole bacterial immunogen on the agarose gel (Table .1). In the first group significant differences ($p< 0.01$) in IgM concentration means at second week and one month post immunization were observed. The IgM concentration reached 89.80± 4.77ng/ml then elevated to 288. 95± 14.92ng/ml; subsequently declined after the third weeks to 138± 6.8ng/ml and 68.40± 3.7ng/ml respectively (Figure 3).

Table .1. Shows the means of IgM concentration of immunized chick embryos with WCS Ag by radial immunodiffusion test.

Time / Groups	IgM concentrations (mg/dl) Mean ± SE	
	Second week	One month
Treatment group(WSC-Ag)	298,3±14.92 Aa	204,8± 6.81 Ba
Control group	5.00±22,87 Ac	13.65±2.74 BC

Figure.1. Shows the inoculation method of egg embryos

All chicken embryos injected with WSC Ag and control group challenged with 3ID50 after 4 weeks post immunization exhibited moderate elevation in the body temperature, which persisted for 2-3 days with mild signs of illness and depression without diarrhea and returned to normal condition within 4 days.

The control group reveals no previous clinical signs but sudden mortality was appeared. Bacteria was isolated from blood and intestine samples on eosin methylene blue agar and the isolates were identified by their morphology, growth characteristics, and Gram staining nature and other confirmation tests.

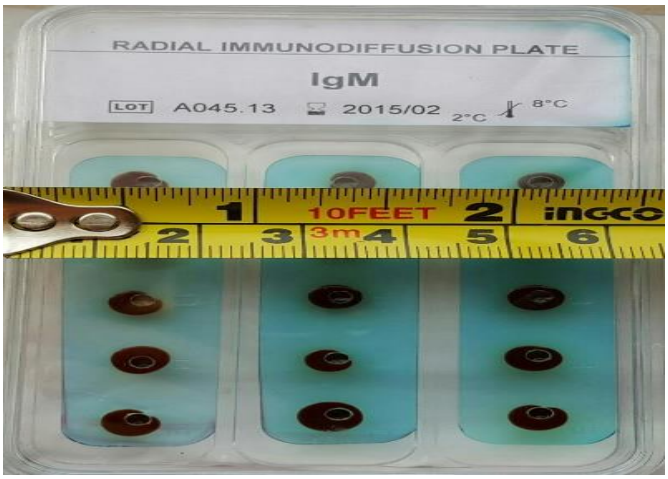

Figure 2. Shows the precipitation zone of Single Radial Immunodiffusion test (Measurement of Single Radial Diffusion assay).

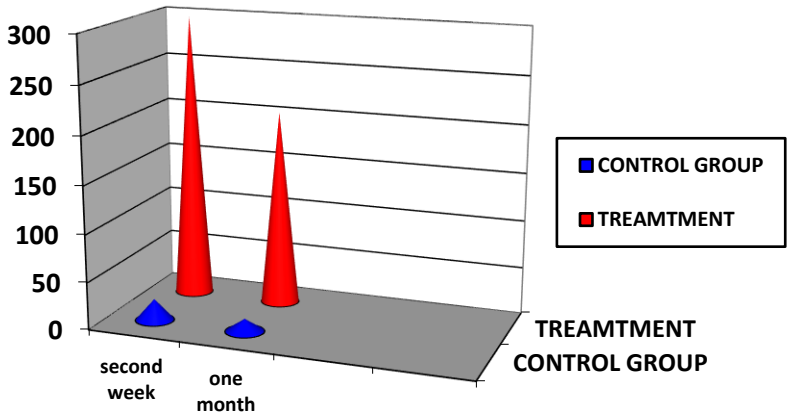

Figure .3. The graph shows the resulting calibration curve.

Discussion

Extra-intestinal habitats infection occur due to infection with avian pathogenic *Escherichia coli* (APEC), and human uropathogenic *E. coli* (UPEC), (Ewers *et al.,* 2007). Ewers *et al.,* (2007) were screened a collection of 526 strains of medical and veterinary origin of various O-types of *E. coli* reference collection (ECOR) group and virulence gene patterns. Moreover, they concluded that certain APEC subgroups consider to be as potential zoonotic agents.

The results of the present study revealed the infectivity of *Escherichia coli* in the control group. This result is compatible with previous studies that reported in the middle region of the Korean peninsula on colibacillosis in layer chickens in 2 commercial egg-producing farms (Oh *et al*., 2011). Oh *et al.,* (2011) found no previous clinical signs in the infected farms but sudden mortality was reached (2.7-4.0%), with severe septicemia and fibrinous polyserositis.

In addition, Mitchell *et al.*, (2015) reported the yolk sac infection in chicks and poultries due to *E. coli*, which were associated with primary infection due to exposure to the bacterium during the hatching process (Stacy *et al*., 2014) evaluated also the prevalence of EXPEC on shell eggs and compared virulence-associated phenotypes between EXPEC and non-EXPEC isolates from both chicken meat and eggs.

Single radial immune-diffusion test has been used to determine the immune-response after vaccination in different diseases such as, viral diseases (Williams, 1993); rabies (Ferguson and Schild 1982; Ferguson, 1986) and human influenza vaccines (Philip and Minor 2015). In this study, SRID results revealed the elevation of IgM concentration in the immunized chick embryos**.** This result is compatible with the results of the assay that reported in previous studies (Ferguson and Schild 1982; Ferguson, 1986). In conclusion, this study approved that the whole cell sonicated uropathogenic *Escherichia coli* antigen (WCS Ag) was able to protect the chick embryos against colibacillosis.

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