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Pathogenicity Testing of Several APEC Isolates Obtained from Naturally Infected Broiler Birds Reared in Basrah

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Abstract: The present study was conducted to investigate the *in vitro* and *in vivo* virulence of several *E. coli* isolates which have been obtained from naturally infected broiler birds. Fifty seven dead and other morbid birds were collected from Al-Basra markets and Basra Veterinary Hospital. A total of 140 sterile cotton swabs from fibrinous pericarditis, fibrinous perihepatitis and airsacculitis were taken and subjected for bacteriological examinations. The result revealed that the overall identification rate of *E. coli* was (37.5%). To differentiate between pathogenic and nonpathogenic isolates, several *in vitro* and *in vivo* pathogenicity testing were performed. Congo Red binding activity showed that 60% were positive, whereas motility test displayed that 51.85% were motile. The ability of isolated *E. coli* to produce hemolysin was found that 44.6% of these isolates were hemolytic. Hemagglutination test indicated that 3.70% of the present isolates were positive. The results of *in vitro* testing indicated that 3 isolates were classified as highly, moderately and slightly virulent according to their characteristics of pathogenicity. *In vivo*, one day old chick lethality test indicated that almost all the three tested *E. coli* isolates which had been inoculated S/C were caused mortality of these chicks within 12-96 h. Three weeks old broiler birds which have been intratracheally inoculated with *E. coli* were clearly displayed characteristic pathological lesions ranging from typical double sided airsacculitis, fibrinous pericarditis and fibrinous perihepatitis to mild one sided airsacculitis.

Key words: Infected broiler birds, airsacculitis, poultry industry

INTRODUCTION

Escherichia coli infections are of significant concern to the poultry industry. It is one of the most frequently encountered important bacterial avian pathogen causing a wide variety of disease syndrome in farmed birds causing 5-50% mortality in poultry industry. Several strategies have been adopted to characterize isolates of *E. coli* and to aid the identification of pathogenic strains (Amitha *et al.*, 2005).

Its pathogenicity has been correlated with numerous extrinsic and intrinsic bird-related factors and conditions. The extrinsic factors include environment, exposure to other infectious agents, virulence and levels and duration of exposure. Intrinsic factors affecting susceptibility to infection include age, route of exposure, active and passive immune status and breed and strain of chicken (Gross, 1990).

Colibacillosis in mammals is primarily an enteric disease whereas in poultry it causes typical localized or systemic disease occurring mostly secondarily when host defense have been impaired. It is characterized by an acute form causing septicemia, resulting in sudden death, while its subacute form characterized by polyserositis resulting in huge mortality (Dhama *et al.*, 2013).

Microbial characteristics associated with virulent avian *E. coli* include antibiotic resistance (Chulasiri and Suthienkul, 1989) production of colicins and siderophores (Dho and Lafont, 1984), type 1 pili (Emery *et al.*, 1992), plasmids (Cavalieri *et al.*, 1984), motility (Elwell and Shipley, 1980) hemolytic reaction (Vidotto *et al.*, 1990) and embryo lethality (Fantannatt *et al.*, 1994). The purpose of the present study was to examine, *in vitro* and *in vivo* virulence of several isolates of *Escherichia coli* obtained from naturally infected boiler birds using one day and 3 weeks old *in vivo* chick lethality and to correlate it with other *in vitro* virulence factors associated with pathogenic avian *E. coli*.

MATERIALS AND METHODS

Fifty seven dead and other morbid broiler birds which exhibited respiratory infection were collected from Al-Basra markets and Basra Veterinary Hospital. A total of 140 sterile cotton swabs from fibrinous pericarditis, fibrinous perihepatitis, airsacculitis and sometimes lungs of these birds were taken and inoculated in brain heart infusion (BHI) broth and subjected for bacteriological examination in an attempt to isolate *E. coli*. Loopfull from the BHI were plated on MacConkey agar (OXOID) and incubated overnight at 37°C in order to determine lactose fermentation. Isolates were

considered positive to lactose fermentation if pink colonies were grown (Tana *et al.*, 2013). The biochemical identification was performed using the API-20E system (Bio-MEIREUX, France) (Ezz El Deen *et al.*, 2010).

In vitro pathogenicity testing was performed by congo red dye binding test. Trypticase soy agar supplemented with 0.003% congo red dye (BDH, UK 3) and 0.15% bile salts (Himedia Manual, 2003) was used for this purpose. Each isolate was cultured on a separate plate and incubated at 37°C. After 24 h incubation, the cultures were left at room temperature for 48 h to facilitate annotation of results. Appearance of red colonies was recorded as congo red (CR+) positive and colonies that did not bind the dye and remained white or grey were considered as congo red (CR -) negative (Sharda *et al.*, 2010).

Motility test medium was prepared according to Himedia manual directions by adding 10 g, tryptose, 5 g, sodium chloride and 5 g, agar to 1 L of distal water and dissolved by heating until boiling (Himedia Manual, 2003). pH was adjusted to 7.2, the medium poured in screw caps bottles and sterilized by autoclaving and used immediately. Motility test medium was inoculated with bacterial culture by straight inoculating needle making a single stab about 1-2 cm down into the medium. The motility was examined after incubation at 37°C for 18 h. Motility was indicated by the presence of diffuse growth away from the line of inoculation and seen to spread from point of inoculation into the agar as a paint brush (Abdullahi, 2010).

For hemolysis assay, *E. coli* isolates were propagated on blood agar base supplemented with 5% washed human blood erythrocytes. Blood agar plates then incubated at 37°C for 24 h and colonies producing clear zones of haemolysis were then recorded as hemolysin positive (Fakruddin *et al.*, 2012; Heller and Drabkin, 1977).

Hemagglutination test was performed as described by Maheswari *et al.* (2013). Briefly, presence of type 1, P fimbriae was detected by the occurrence of hemagglutination in the presence or absence of mannose, respectively. The hemagglutination was detected by clumping of erythrocytes by fimbriae of bacteria in the presence of D-mannose. This test was carried out as per the direct bacterial hemagglutination test (slide method) and mannose-sensitive (MSHA) and mannose-resistant (MRHA) hemagglutination. The strains of *E. coli* were inoculated into 1% nutrient broth and incubated at 37°C for 48 h for full fimbriation. Human blood group "O" red blood cells were washed thrice in normal saline and made up to a 3% suspension in fresh saline. They were used immediately. The test was carried out on a multiple-

concavity slide. One drop of the red blood cells (RBCs) suspension was added to a drop of the broth culture and slide was rocked to and fro at room temperature for 5 minutes. Presence of clumping was taken as positive for hemagglutination. MSHA was detected by the absence of hemagglutination in a parallel set in which a drop of 2% w/v D-mannose (S.d. Fine-Chem limited, Mumbai) was added to the red cells and a drop of broth culture. MRHA was detected by the presence of hemagglutination of 3% "O" group of human RBCs in the presence of 2% mannose.

In vivo pathogenicity testing was conducted according to Catherine *et al.* (2012). According to the results of *In vitro* testing, high, moderate and mild (slight) virulent isolates were selected; therefore three groups, each with 5 one-day-old chicks were inoculated subcutaneously with 0.5 mL 10⁸ CFU of an overnight culture in LB-Miller broth without agitation and the mortality was recorded 4 days post-inoculation. Positive and negative control groups were also used. The positive group was inoculated with sterile nutrient broth while the negative group remained uninoculated. Strains were classified as pathogenic when at least one chick is died. Nonpathogenic avian *E. coli* strain was also used as a confirmative control.

On the other hand, thirty 3 weeks old broiler chicks were also used to assess the pathogenicity of the same isolates by intra-tracheal inoculation of 0.5 mL 10⁸ CFU (Ewers *et al.*, 2008). The birds were divided into 6 groups, the first one consisted of 7 birds and each of the other five groups consisted of five birds.

The live birds were killed on day 14 and subjected to post-mortem examinations (Permin *et al.*, 2006).

RESULTS AND DISCUSSION

Escherichia coli is a commensal organism inhabiting human and animal intestinal tract and can cause a variety of extra-intestinal infections when enters into unnatural sites (Sharma *et al.*, 2007). The ability of *E. coli* to cause extra-intestinal infections depends largely on several virulence factors which help to survive under adverse conditions. Identification of virulence factors is important for understanding bacterial pathogenesis and their interactions with the host, which may also serve as novel targets for drug and vaccine development (Banu *et al.*, 2011).

According to the standard procedures of isolation and identification, the present study revealed that the overall identification rate of *E. coli* isolates was 45/120 (37.5%). This result was in agreement with that of Ahmad *et al.* (2009) who isolated pathogenic *E. coli* from drinking water of poultry farms with incidence of 30.24 % and Rajaa (2013) who isolate pathogenic *E. coli* O78:K80 serotype from broiler chicks with an incidence of 46%. On the other hand the result of the present study

disagree with that of Ezz El Deen *et al.* (2010), who isolated *E. coli* from chickens with incidence of 75%. These variations may be either due to the site of sample collections or geographical situations.

To differentiate between pathogenic and nonpathogenic *E. coli* which have been isolated in the present study, CR binding activity test was performed. Many researchers advocated the use of CR dye with the objective of distinguishing between pathogenic and non-pathogenic microorganisms (Berkhoff and Vinal, 1985; Stebbins *et al.*, 1992). The result showed that 27 out of 45 (60%) isolates were CR+. This result was in agreement with that of Ahmad *et al.* (2009), who isolated pathogenic *E. coli* from water sources and poultry drinkers on different poultry farms in Pakistan by the use of CR medium with an incidence of 77%. On the other hand, this result disagree with that of Ezz El Deen *et al.* (2010) and Rajaa (2013) who stated that 100% and 26.08% of *E. coli* isolates from diseased chickens was CR+, respectively.

Capacity of *E. coli* to produce multiple virulence factors may contribute to its pathogenicity in extra-intestinal infections (Sharma *et al.*, 2007). These virulence factors enable some members of the normal flora to elicit an infection by overcoming the host defense mechanisms (Emody *et al.*, 2003). Previous studies have demonstrated that APEC possess virulence properties which promote the bacterial colonization and tissue invasion, subsequently leading to the colibacillosis development (Tana *et al.*, 2013).

The result of motility of the present study indicated that 14/27 (51.85%) of *E. coli* isolates were motile. Heller and Drabkin (1977) were used motility test to determine the pathogenicity of *E. coli*, whereas Zinnah *et al.* (2007) were use this test for characterization of *E. coli* isolated from samples of different biological and environmental sources.

The present study showed that 10/27 (37.03%) of *E. coli* isolates were able to produce hemolysis. This result was in agreement with Fakruddin *et al.* (2013) who found that (44.6 %) of clinical isolates of *E. coli* were hemolytic. Production of hemolysin usually associated with pathogenicity of *E. coli* and especially responsible for more severe forms of infections (Johnson, 1991). On the other hand, the result of the present study disagree with that of Brenda *et al.* (1993) who isolated 44 *E. coli* strains from cases of avian colibacillosis and non of these tested strains appeared to produce hemolysin.

For hemagglutination assay, the present study demonstrated that only 1/27 (3.70%) of isolates was positive. This result disagree with those of Maheswari *et al.* (2013) and Fakruddin *et al.* (2013) who stated that (100%) and (47.7%) of *E. coli* isolates were able to induce hemagglutinin, respectively.

The adhesive ability of an enteropathogen is usually assessed by determining the hemagglutinating ability, because the erythrocyte membrane is believed to possess the homologous of the mucosal substances involved in bacterial adherence to epithelial cells (Hager *et al.*, 2010).

Sharda *et al.* (2010) used CR, hemolysis and agglutination assays for in vitro pathogenicity testing of *E. coli* isolated from poultry and found that 95.38, 0 and 70.75% were MRHA positive and 20% were MSHA positive. These results were in disagreement with the results of the present study.

One-day-old chick lethality test of the present study indicated that almost all the three tested *E. coli* clinical isolates were caused mortality of one-day-old chicks. Death of the chicks occurred within 12 to 96 h following inoculation of bacteria. The mortalities of the highly, moderate and slightly virulent isolates were 100, 80 and 60%, respectively, whereas no mortality was recorded in both positive and negative control groups. According to Zinnah *et al.* (2007) all the *E. coli* isolates of the present study were considered to be virulent as they caused more than 50% mortality in the one-day-old chicks following inoculation. These findings were contradict with those of Ngeleka *et al.* (2002) who recorded a variable degree of virulence ranging from high to moderate. This may be due to difference in the route of inoculation and bacterial concentration of the inoculum. Catherine *et al.* (2012) were used one day old chicks lethality test as a model for pathogenicity testing of *E. coli* isolates.

Characteristic pathological lesions in the broiler chicken, which were intra-tracheally inoculated with *E. coli* were clearly observed in this study as shown in Table 1 above. These findings were previously recorded by Dho-Moulin and Fairbrother (1999) in experimentally and naturally infected broiler chickens with *E. coli*.

Although, the infected birds in the present study were not weighed, but a clear retarded growth was observed in comparison with the control uninfected birds. This result was in accordance with that of Dho-Moulin and Fairbrother (1999) who recorded lower weight gains in the infected birds. Michael and Angesia (2008) were used 3 weeks old bird lethality test as a model to determine the pathogenicity of *E. coli* isolates obtained from chickens.

From the data obtained in this study, it seemed probable that CR binding activity, haemolysin production, MRHA of human type O erythrocytes and motility might be the important characteristics that enabled *E. coli* to cause extra-intestinal infections. Study on other characteristics might reveal the virulence determinants of the isolates that were otherwise negative for all the characteristics studied.

Table 1: Results of 3 week old birds lethality test with 3 different *E. coli* isolates

Degree of virulence of isolates according to <i>in vitro</i> testing	No. of dead and mortality rate (%)	Post-mortem findings
Highly virulent	(2) 20%	3 birds with typical double sided airsacculitis, fibrinous pericarditis and fibrinous perihepatitis 2 birds with mild airsacculitis, fibrinous pericarditis and fibrinous perihepatitis 2 birds with no pathological findings
Moderate	10%	2 birds with typical double sided airsacculitis, fibrinous pericarditis and fibrinous perihepatitis 2 birds with mild one sided airsacculitis and fibrinous perihepatitis 1 bird with no pathological findings
Slightly (mildly) virulent	10%	2 birds with typical both sided air sacculitis, fibrinous pericarditis and fibrinous perihepatitis 3 birds with no pathological findings
Non pathogenic isolate	0%	4 birds with no pathological finding 1 bird with one sided airsacculitis and fibrinous pericarditis
Positive control (Nutrient broth)	0%	2 birds with cloudy airsacculitis 3 birds with no pathological findings
Negative control (Uninoculated)	0%	5 birds with no pathological findings

Each group was consisted of 5 birds, except group 1 consisted of 7 birds

Further study involving a large number of *E. coli* isolates obtained from naturally infected birds with septicemia and other subacute polyserositis would be necessary before any factor could be implicated for virulence of *E. coli* infection at the extra-intestinal sites, although one day and 3 weeks old lethality testing clearly revealed the virulence characteristics of the studied isolates.

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