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Original Research Article

Phenotypic characterization of *Escherichia coli* isolated from broiler chickens

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ABSTRACT

Colibacillosis is one of the most important diseases of chickens, resulting in significant losses. The current study aimed to investigate the prevalence of *E. coli* **infections** in broiler chickens detecting their phenotypic characters such as Congo red binding activity, serum resistance and **proteolytic activities**. Samples were collected from 297 broiler chickens of different ages from different farms in El-Fayoum Governorate during the period from April 2017 up to March 2018. Bacteriological examination showed that 98 *E. coli* isolates were recovered with a prevalence rate of 33%. Results of Congo red binding activity and serum resistance revealed that all *E. coli* isolates (100%) showed Congo red binding activity and survive for 1 and 6 hrs and grown for 18 hrs in the presence of serum. Results of proteolytic activity revealed that 43 *E. coli* isolates (43.9%) were able to digest casein of skimmed milk while 16 isolates (16.3%) were positive for gelatin liquefaction test.

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INTRODUCTION

Colibacillosis refers to any localized or systemic infection caused entirely or partly by avian pathogenic *E. coli* (*APEC*), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), cellulitis, swollen-head syndrome, peritonitis, salpingitis, panophthalmitis, and omphalitis/yolk sac infection **(Saif *et al.*, 2003)**. Colisepticemia is the most common form of colibacillosis and is responsible for significant economic losses in aviculture in many parts of the world **(Ewers *et al.*, 2003)**.

Primary infection is most commonly via the respiratory tract and is usually secondary to a mycoplasma or viral infection **(Ramirez *et al.*, 2009)**. The disease inducing potential of these isolates has been explained by the natural event of specific virulence factors. Many virulence factors have been associated with avian pathogenic *E. coli* (APEC) strains, although their role in the pathogenesis is not well known **(Mellata *et al.*, 2003)**.

Serum resistance is one of the most important pathogenicity mechanisms of APEC strains. Virulence factors that increased bacterial resistance to serum and colonization of internal organs of infected chickens were O_{78} LPS and the K1 capsule of *E. coli* **(Mellata *et al.*, 2003)**.

There is a strong correlation between the expression of the Congo red phenotype and virulence in avian *E. coli* **(Gjessing and Berkhoff, 1989)**.

***APEC* produces a cytotoxin cause similar morphological changes in target cells** and **are similar with respect to heat lability and susceptibility to proteolytic enzymes. Proteolysis is considered to be a virulence factor (Salvador *et al.*, 2001).**

The purpose of this study was to investigate the prevalence of *E. coli* **infections** in broiler chickens detecting their phenotypic characters such as Congo red binding activity, serum resistance and p**roteolytic activities**.

MATERIAL AND METHODS

2.1. Chickens Samples.

Samples were collected from 297 broiler chickens of different ages (3-5 weeks) from different farms in El-Fayoum Governorate during the period from April 2017 up to March 2018. These chickens were suffering from respiratory manifestations (Coughing, sneezing, ralles, nasal discharge and sometimes swelling of infra orbital sinuses either bilateral or unilateral). These chickens were subjected to clinical and postmortem examinations. Samples were collected from the lesions in the internal organs including pericardium, liver, air sac and kidneys according to naked eye lesions

2.2. Bacteriological isolation.

For isolation of *E. coli*, the specimens were inoculated into MacConkey's broth and incubated aerobically at 37ºC for 24 hrs. Then, a loopful of this culture was streaked out onto MacConkey's agar and incubated at 37ºC for 18-24 hours. Lactose fermenter (pink) colonies were streaked onto and eosin methylene blue agar and confirmed as *E. coli* using the standard biochemical tests according to **Collee *et al.* (1996)**.

All the recovered isolates were identified morphologically and biochemically according to **Collee *et al.* (1996)** and **Quinn *et al.* (2002)** using the following tests; oxidase, TSI, indole production, citrate utilization and motility test.

2.3.2. Identification by using API20E kit.

The appropriate API kit (API20E, Oxoid) was used. API strips should only be used to identify pure cultures. It was used according to the manufacturer's instruction.

2.3.3. Detection of virulence factors of *E. coli* isolates:

All *E. coli* strains were tested for detection and evaluation of its virulence factors.

2.3.3.1. Congo red binding assay.

E. coli isolates were tested for its growth status on Congo red medium after incubation for 24 hrs at 35°C then left at room temperature for additional 2 days (not to exceed 4 days) **(Berkhoff and Vinal, 1986)**. Congo red positive (CR^+) *E. coli* was indicated by the development of red colonies. Congo red negative (CR^-) *E. coli* did not bind the dye and appeared as white colonies.

2.3.3.2. Serum resistance assay.

About 0.05 ml from cell suspension equal to 2.5×10^8 *cfu*/ml HBSS was add to the same amount of serum and incubated at 37°C. Then, 10 µl were plated on Muller Hinton agar 0 min and 180 of incubation, the plates were further incubated at37°C. Susceptibility of bacteria to serum bactericidal activity expressed as percentage of bacteria surviving after 180 min and overnight incubation in relation to the original growth of bacteria at 0 min in the controls **(Siegfried *et al.*, 1995)**.

2.3.3.3. Tests used for proteolytic action.

2.3.3.3.1. Proteolytic action on skimmed milk agar.

E. coli strains cultured in tryptic soy broth (TSB) were centrifuged at 12000g for15 min. at 4°C and filtered through a Millipore 0.45 µm pore-diameter syringe filter. Clarified supernatant was tested for proteolytic activity on casein agar plates. Casein agar plates consisted of 25 mM Tris (pH7.2), 150 mM NaCl, 0.6% casein (Sigma technical grade) and 1% Bacto Agar (Difco). Aliquots (10µl) of culture supernatants were placed in 3 mm diameter wells cut in the casein agar and incubated at 37°C for 18hrs. The plates were overlaid with 3% acetic acid, and proteolytic activities were noted as a zone of clearing around the sample well. One µg/ml trypsin was used as a positive control. Caseinolytic activity of the proteolytic bacteria, also relative to trypsin used as a control was determined by measuring the diameter of the proteolytic zones around each well **(Martley *et al.*, 1970)**.

2.3.3.3.2. Gelatin liquefaction test.

A heavy inoculum of tested proteolytic bacteria (18-24hrs old was inoculated by stabbing 4-5 times (half inch) on the tube containing nutrient gelatin medium then incubated (the inoculated tube along with an un inoculated medium) at 35°C for up to 2 weeks. the tubes were remove daily from the incubator and placed at refrigerator (4°C) for 15-30 min. (until control is gelled) every day to check for gelatin liquefaction. Gelatin normally dissolves at 28°C and above and so to confirm that liquefaction was due to gelatinase activity, the tubes are kept in refrigerator at 4°C). The tubes were tilted to observe in gelatin has been hydrolyzed. Positive test: partial or total liquefaction of inoculated tube **(Collee *et al.*, 1996)**. Negative result: complete solidification of the inoculated tube even after exposure to cold temperature.

RESULTS

3.1. Prevalence of *E. coli* infections in broiler chickens.

Out of 297 broiler chickens, 98 bacterial isolates were recovered with a prevalence rate of 33%. *E. coli* isolates were isolated recovered from different organs as follow: liver (31), kidney (18), air sacs (20) and pericardial sac (29) as 31.6%, 18.4%, 20.4% and 29.6%, respectively (Table 1).

Table (1): Prevalence of *E. coli* isolation from different organs of broiler chickens.

Site of isolation	*E. coli*	
	No	%
liver	31	31.6
kidney	18	18.4
Air sacs	20	20.4
Pericardial sac	29	29.6
Total No. of isolates	98	100

%: was calculated according to the total number of isolates.

3. 2. Detection of some virulence factors of *E. coli*:

3.2.1. Detection of Congo red binding activity:

Results of Congo red binding activity revealed that all *E. coli* isolates (*n*=98; 100%) showed Congo red binding activity (CR+) **(Fig. 1)**.

3. 2.2. Serum resistance assay:

Results of serum resistance also showed that all *E. coli* isolates (*n*=98; 100%) were able to survive for 1 and 6 hrs and grown for 18 hrs in the presence of serum **(Fig. 2)**.

3.2.3. Prevalence of proteolytic *E. coli* isolates.

3.2.3.1. Proteolysis of skimmed milk:

Results showed that 43 *E. coli* isolates (43.9%) were able to digest casein and surrounded by clear zones around well **(Table 2& Fig. 3)**.

3.2.3.2. Gelatin liquefaction test:

Out of 98 *E. coli* isolates, 16 were positive for gelatin liquefaction test (16.3%) **(Table 2 & Fig. 4)**.

Table (2): Proteolytic action of *E. coli* recovered from broiler chickens.

Biochemical Test	*E. coli* (*n*=98)	
	No. of positive isolates	%
Skimmed milk hydrolysis test	43	43.9
Gelatin liquefaction test	16	16.3

%: was calculated according to the number of isolates (*n*=98).

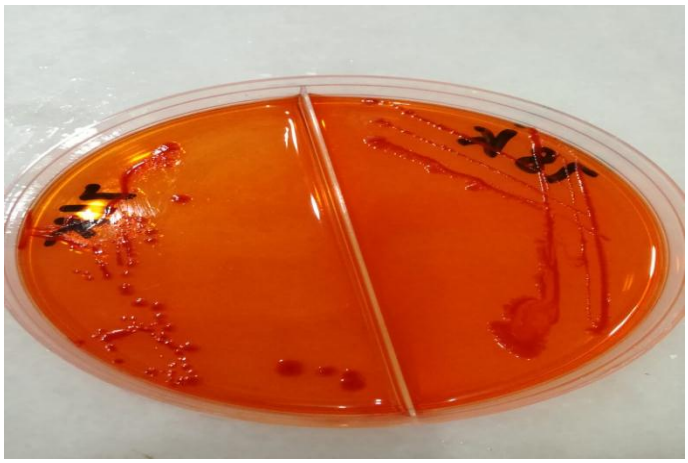

Fig. (1): The Congo red binding activity of *E. coli* isolates. Positive colonies appear red.

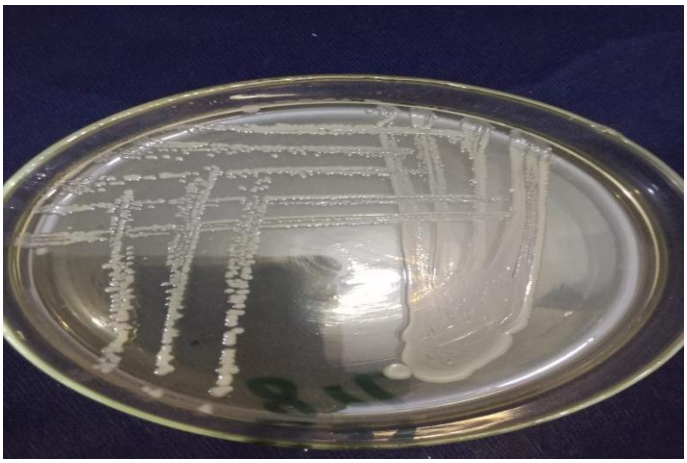

Fig. (2): growth of *E. coli* isolates in the presence of serum.

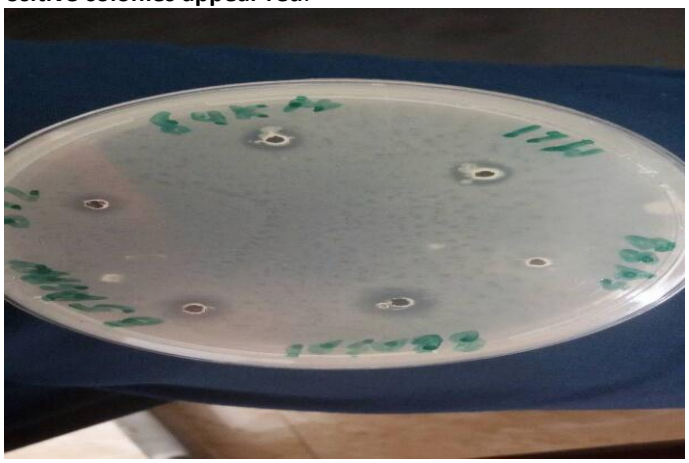

Fig. (3): Caseinolytic activity of the proteolytic *E. coli* determined by measuring the diameter of the clear zones around each well.

Fig. (4): proteolytic effect of E. coli on gelatin medium.

DISCUSSION

Avian colibacillosis is one of the most important diseases of chickens, resulting in significant losses among baby chicks, broilers and laying hens **(Ibrahim *et al.*, 1998)**. This syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis being most typical **(Ewers *et al*., 2003)**.

In the current study, the prevalence of *E. coli* isolation from the internal organs (pericardium, liver, air sac and kidneys) of broiler chickens were detected in a total of 297 broiler chickens **(Table 1)**. The prevalence of *E. coli* isolation was 33%. Nearly similar results were recorded by **Gomis *et al.* (2000)**; who isolated *APEC* at a percentage of 32%, **Mohamed *et al.* (2014**); 34.8%, and **Younis *et al.* (2017)**; 36.5%. On the other hand, a higher recovery rate; 92%, was recorded *by* **Dandachi *et al.* (2018)**.

Regarding *E. coli* isolation from different organs, the results illustrated in table (1) showed a higher percentage of isolation from liver (31.6%), followed by pericardial sac (29.6%), air sacs (20.4%) and finally kidney (18.4%). **Mohamed *et al.* (2014**) recovered *E. coli* from extra intestinal origins (liver, heart blood, lung, trachea, and sinus) and from cases of swollen head syndrome. **Paixao *et al.* (2016)** isolated 66 APEC from liver and spleen lesions as well as ascitic fluid of broiler breeders with clinical signs of colibacillosis. **Younis *et al*. (2017)**

showed the recovery rate of *E. coli* from different chicken samples; lungs, spleen, heart, liver, and intestinal contents as 28.76%, 27.39%, 23.28%, 15.06%, and 5.46%, respectively. **Zainal *et al.* (2013)** isolated *E. coli* from Heart and spleen (26% of each) which were the most common samples positive for *E. coli* followed by the liver (22%), air sacs (17%) and peritoneal swabs (9%).

E. coli that cause infections usually possess one or more virulence properties that may help in establishment the infection. Among these factors, Congo red binding activity, haemagglutination, haemolytic activity and toxin production are the most commonly studied by **(Berkhoff *et al.*, 1986)**. Moreover, **Ewers *et al.* (2005)** studied many virulent properties such as haemolytic activity, Congo red uptake, haemagglutination activity, hydrophobicity (salt agglutination activity), serum resistance, enterotoxigenic and verotoxigenic activities, invasiveness property and production of heat stable toxins. Also, **Mellata *et al.* (2003)** showed the variety of virulence factors implicated in promoting the extra-intestinal diseases in avian species including adhesions, iron acquisition systems, haemolysins, anti-bactericidal factors (outer membrane protein A, LPS, K1-capsule, and colicin production), and toxins (heat stable toxin and flagella toxin). Also, P fimbriae, curli, aerobactin, K1 capsule and temperature-sensitive haemagglutinin were included. Moreover, **Mellata *et al.*** (**2003)** detected virulence factors that increased bacterial resistance to serum and colonization of internal organs of infected chickens, serum resistance is one of the most important pathogenicity mechanisms of APEC strains.

In the current, results of Congo red binding activity and serum resistance revealed that all *E. coli* isolates (*n*=98; 100%) showed Congo red binding activity (CR+) and were able to survive for 1 and 6 hrs and grown for 18 hrs in the presence of serum **(Photo. 1& 2)**. **Berkhoff and Vinal (1986)** sowed the direct correlation between the ability of clinical isolates of to bind Congo red dye (CR) and their ability to cause septicemic infection in chickens, congo-red-positive colonies were isolated from air sacs, pericardium, liver, lung, joint fluid, and heart blood of chickens with lesions of colisepticemia, preliminary findings suggest that the CR dye binding used as a phenotypic marker to differentiate between invasive and noninvasive isolates.

In this study, **table (2)** showed the proteolytic action of *E. coli* which recovered from examined chickens with colisepticaemia, by measuring the diameter of the clear zones around each well on skimmed milk media, 43 *E. coli* isolates (43.9%) were able to digest casein and surrounded by clear zones around well , in another study screened the proteolytic activity of the colonies on a skim milk agar plate.**(Callan *et al.*, 1997)** were tested for proteolytic activity on casein agar plates, proteolytic activity was noted as a zone of clearing around the sample well. by measuring the diameter of the proteolytic zones around each well. **Vijayaraghavan *et al.* (2013)** invested the proteolytic activity by using bromocresol green dye on substrate agar plates, to determine the proteolytic activity by flooding bromocresol green reagent on casein/skimmed milk agar plates. A zone of proteolysis was detected on the casein/skimmed milk agar plates. The proteolytic activity was determined as the clear zone where as the rest of the plates were greenish-blue in colour BCG staining is simple and easy to perform. **King *et al.* (2009)** compared the secreted bacterial casein proteolytic activity to that of a known trypsin standard, some bacterial isolates produced white or off-white precipitants or clouding around the inoculum site on the caseinate plates.

By Gelatin liquefaction test out of 98 *E. coli* isolates 16 were positive for gelatin liquefaction test (16.3%). **Quintero *et al.* (2014)** observed differences in a white ring of protein precipitation that occurred strongly on the

gelatin plates by higher concentrations of trypsin (2.5mg/ml) were used and only slightly on the casein plates when treated with the same trypsin concentration.

CONCLUSION

It was concluded that colibacillosis is one of the most important diseases of chickens, resulting in significant losses. *E. coli* has many virulent properties such as Congo red uptake, serum resistance and proteolytic activities.

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