Research Article

In vitro and *In vivo* Pathogenicity tests of Local Isolates APEC from Naturally Infected Broiler in Baghdad

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Abstract

This study was conducted to investigate the *in vitro* and *in vivo* virulence of *E. coli* isolates obtained from naturally infected broiler birds. Two hundred morbid birds were collected from Baghdad private veterinary clinics in interval January-June/ 2015 samples were taken and subjected for bacteriological examinations. The result revealed that the overall identification rate of *E. coli* was (45%).To differentiate between pathogenic and nonpathogenic isolates, several *in vitro* and *in vivo* pathogenicity testing were performed. Congo Red binding activity showed that 60.4% were positive, whereas, the ability of isolated *E. coli* to produce hemolysin was found that 53.3% of these isolates were hemolytic. While acriflavine test was preformed, The results showed that 88.24 % isolates were negative to acriflavine test i.e. pathogenic then serotyping was done and the results revealed that 47 % were identified as *E.coli* serotype 02, 33 % were identified as *E. coli* serotype 078 and 20% were 01 serotype . *In vivo*, one day old chick lethality test indicated that almost all the three tested *E. coli* isolates which had been inoculated intranasally were caused mortality of these chicks within 12-96 h., fourteen 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 fibrinousperihepatitis to mild one sided airsacculitis.

Keywords: *In vitro* and *In vivo* virulence, *E. coli, In vivo* pathogenicity testing, acriflavine test.

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).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 (Emeryet al., 1992), plasmids (Cavalieri et al., 1984), motility(Elwell and Shipley, 1980) hemolytic reaction (Vidotto etal., 1990) and embryo lethality (Fantannatt et al., 1994). The purpose of the present study was to examine, invitro 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.*

private veterinary clinics in interval January-June/ 2015 . Sterile cotton swabs from fibrinous pericarditis, fibrinousperihepatitis as in Fig. (1), airsacculitis, lungs and liver.

Materials and Methods

1. Samples collection

Two hundreds broiler birds which exhibited respiratory infections were collected from Baghdad
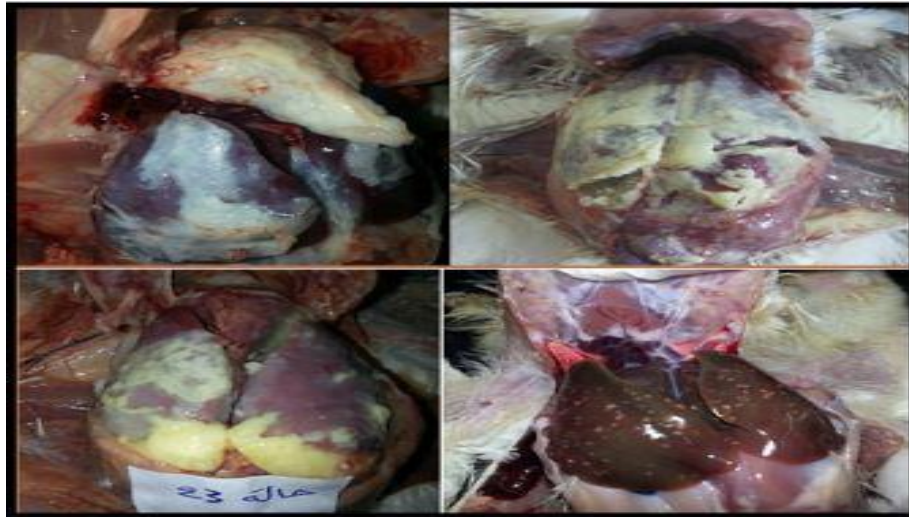

Fig.1: morbid birds suspected with coli bacullosis

Samples from these birds were taken and inoculated in Brain Heart Infusion (BHI) broth and subjected for bacteriological examination to isolate *E. coli*, APEC contributes significantly to first week mortality in chickens, turkeys and web-footed birds (Gregersen et al., 2010).

2. Initial isolation of *E. coli*

Loop full from BHI broth were plated on MacConkey agar and incubated overnight at 37 C to determine lactose fermentation. Isolates were considered positive to lactose fermentation if pink colonies were observed . Characteristic colony morphology of the organisms indicating the features of *E. coli* was selected to subculture on EMB agar plates and incubated at 37ºC for 24 hours (Jorgensen and Pfaller, 2015).

3. Identification of *E. coli*:

A. Morphological characteristics:

For studying morphological characteristics of the isolates by naked eye, the morphology of the well growing organisms on MacConkey and EMB agar was considered (Beutinet al., 1996).

Microscopic properties of the isolated bacteria with Gram stain were also studied through examining stained slide under light microscope to establish the Gram stain reaction and cellular morphology typical *E. coli* must shows coccoidand red cells (Jorgensen and Pfaller, 2015).

2.Biochemical tests:

According to Jorgensen and Pfaller (2015) the API 20 E was used for confirmation of *E. coli* isolates.

4. Detection of the virulence:

In Vitro pathogenicity testing: This was performed by

A.Congo Red dye binding activity test:

Tryptic Soy agar supplemented with 0.03% Congo Red dye and 0.15% bile salts were used for this purpose. Each smooth isolate was cultured on a separate plate and incubated at 37 C. After 24 hrs of incubation, the cultures were left at room temperature for 48 hrs 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 (Fodor et al , 2010).

B. Hemolysis test:

E. coli isolates were cultivated on blood base agar supplemented with 5% washed sheep blood erythrocytes. Blood agar plates were, then incubated at 37 C for 24 hrs and colonies producing clear zones of hemolysis, which were recorded as hemolysin positive (Fakruddin et al ., 2013)

C. Acriflavinetest

To differentiate between pathogenic and nonpathogenic *E. coli* which have been isolated in the present study, Acriflavinetest was preformed according to Kloryga (1986). The strain that isolate from clinical cases in Baghdad city showed variation in their agglutination strength. Some strains showed negative result (no agglutination) and thus smooth strain are considered pathogenic strains . Other strain that agglutinate showed variation in agglutination speed and size. The strains that agglutinate are rough strain and considered to be slightly pathogenic and cause milled clinical size. The rough strains agglutinate immediately at concentration (1:500) in less than half a minute.

D. Serological tests :

This test was used for serotyping of *E. coli* 01, 02, 078 by using Monovalent *E. coli* antisera (Biovac, France) to detect the somatic antigen. One isolate of each *E. coli* serogroup was selected according to its pathogenicity in vitro (01, 02 , 078) to be used *in vivo* .

3.6.3. *In Vivo* lethality testing:

A.Preparation of inoculum:

For colony forming unit (CFU/ml) count, each isolates was grown in nutrient broth for overnight. Then 10 fold dilutions were made and 0.5 ml of each 10 fold dilution was transferred aseptically to the nutrient agar plate using a fresh pipette for each dilution. The diluted samples were spread on the plate with sterile L-shaped glass spreader. Then the plates were incubated at 37 C for 24 hrs. Following incubation only those plates exhibiting 30 to 300 colonies were counted. For each dilution three plates were used and the mean of three plates were calculated .The number of bacteria per ml of original sample was obtained by multiplying the mean of number of colonies by diluting factor. The results of CFU were expressed as the number of organism per ml of sample (Rahman et al ., 2004). 10^8 CFU/ml was considered in this study (Anta˜o et al., 2008).

B. Broilers for in vivo tests:

Fifty six broiler chicks (Breed : Rose 308, Origin : Belgium) were brought in good condition from AL-jazeera Hatchery-Baghdad) . Twenty eight birds one day old , and 28 birds at 14 days were used :

C. One-day-old chick lethality test:

It was conducted according to Schouler etal., (2012). According to the results of *in vitro* testing , 3 virulent strains 01,02 and 078 isolates (Strains gave CR⁺ , Hemolysis positive and Acriflavine negative) were selected ; therefore 28 birds was divided into 4 groups, 7 birds each, were inoculated intranasal with 0.25 ml of 10^8 CFU/ml of an overnight culture in BHI broth without agitation , and the mortality was watched for 4 days post-inoculation. Control group was remained un-inoculated. Strains (i.e. 01,02 and 078) were classified as pathogenic when at least one chick is died . Nonpathogenic *E. coli* avian strain was also used as a confirmative control.

D. Fourteen days old chick lethality test:

Twenty eight birds at 14 days old broiler were used to assess the lethality of the same isolates by intranasal inoculation of 0.5 ml of 10^8 CFU/ (Anta˜o et al.,2008) .

The birds were divided into 4 groups, 7 birds for each.

The live birds were killed on day 7 post-inoculation and subjected for post-mortem examinations (Permin et al., 2006).

Results and Discussion

1. Isolation and characterization of *Escherichia coli*

According to the standard procedures of isolation and identification, the present study revealed that the overall identification rate of presumptive *E. coli* isolates was 95 out of 200 cases (45%) as shown in Fig.2, Table 1:

Table 1 : Number of Presumptive samples for *E. coli* in collected samples

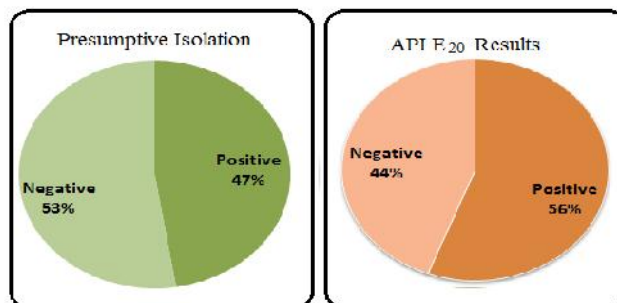| Samples	Number	Positive	Negative
Presumptive Isolation	200	95	105
API E20	95	53	42

Fig 2: Percentage of Presumptive samples for *E. coli*

This result was in agreement with other Iraqi studies (Ali, 2013) who isolated *E. coli* from broiler chicks with an incidence of 46%. On the other hand , the results of the present study was disagreed with that of Giovanardi et al., (2005) , who isolated *E. coli* from broiler chick flocks among the early cases of Colibacillosis with incidence of 100% and 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.

In the present study, post-mortem examination of naturally infected broiler birds displayed pathological changes ranging from mild to severe fibrinous pericarditis , fibrinousperihepatitis and/or airsacculitis. Bacteriological examination of the samples from these fibrinous material ,organs and blood which have been performed on MacConkey agar revealed pink color colonies, which indicated that these organisms were lactose fermented bacteria. On Eosin Methylene Blue (EMB) agar these bacteria exhibited green metallic sheen which could restrict the isolate of *E. coli* (Fig 3) .
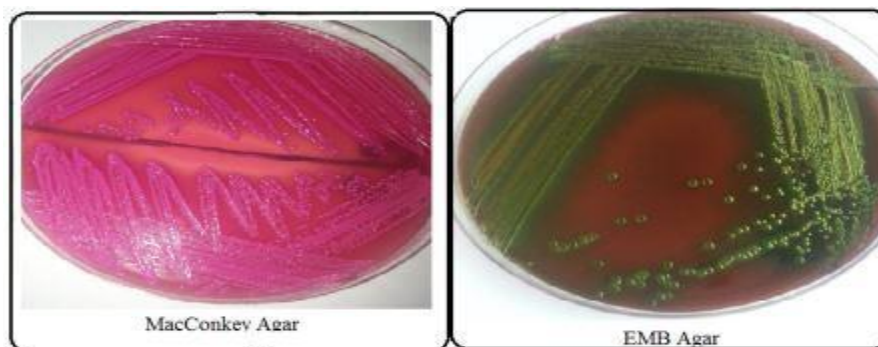

Fig 3 : Reactions of E. coli on MacConkey agar (Left) , and EMB agar (Right)

The Catalase production test and Oxidase Test were carried out, the results as shown in Fig 4 :

Fig 4 : Catalase reaction (Left) , and Oxidase reaction (Right) .

This confirms that these enzymes produce by aerobic grown bacteria.

Microscopic examination of Gram's stained smear of these bacteria revealed Gram negative rod shape as in Fig.5
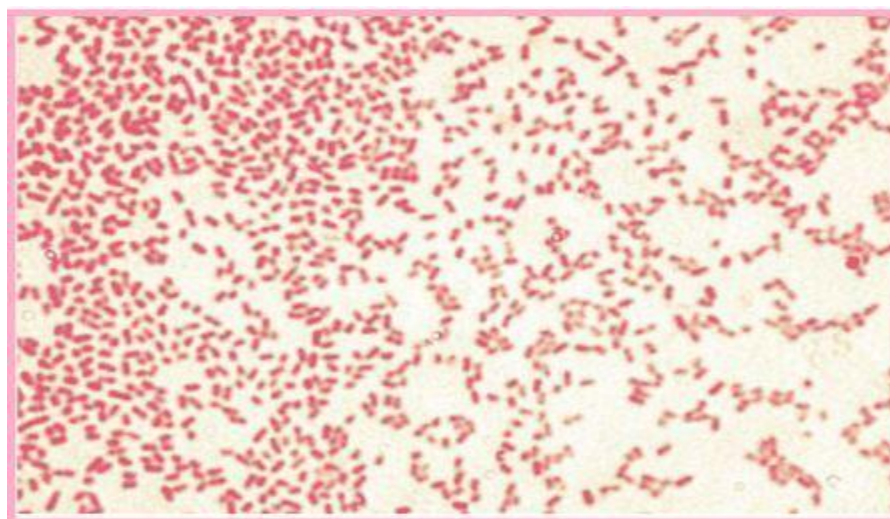

Fig 5 : Gram stain of *E. coli* , short rods or coccoid

These isolates were subjected for API 20E system for confirmation, Table 2 and Fig. 6 . According to these standard procedures of isolation and identification, the present study revealed that the overall identification rate of *E. coli* isolates was 53/95 (56 %).

Fig.6 : *E. coli* reactions using API 20E System

Table (2) : Results of biochemical test on API20 *E.coli*

Test	Result
ONPG (Ortho nitrophenyl ß dgalactopyranosidase)	+
ADH (Arginine dihydrolase)	-
LDC (Lysine decarboxylase)	+
ODC (Ornithine Decarboxylase)	+
CIT (Citrate utilization)	-
H2S (H2S production)	-
URE (Urease)	-
TDA (Tryptophanedeaminase)	-

IND (Indole production)	+
VP (VogesProskauer)	-
GEL (Gelatinase)	-
GLU (Glucose)	+
MAN (Mannitol)	+
INO (Inositol)	-
SOR (Sorbitol)	+
RHA (Rhamnose)	+
SAC (Saccharose)	-
MEL (Melibiose)	+
AMY(Amygdalin)	-
ARA (Arabinose)	+

2. Pathogenicity tests:

4.2. 1. *In Vitro* pathogenicity tests:

A. Congo Red test:

To differentiate between pathogenic and nonpathogenic *E. coli* which have been isolated in the present study, Congo Red binding activity test was performed. The result showed that 32 out of 53 (60.4%) isolates were CR$^+$ (Fig.7).

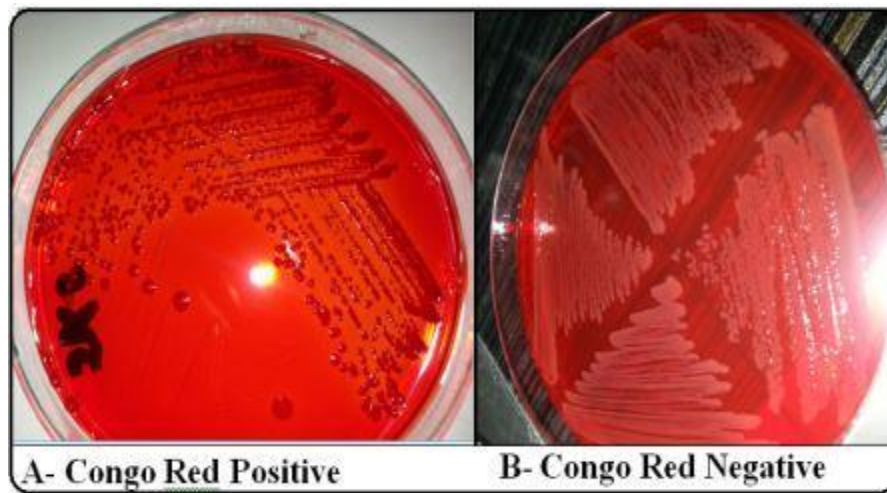A- Congo Red Positive   B- Congo Red Negative

Fig.7 : Reaction of different strains using Congo Red

The results confirm the association between Congo Red binding *E. coli* and colisepticemiain commercial broilers, some researchers considered it as an epidemiological marker to identify the APEC strains, This marker can differentiate pathogenic strains from commensal ones especially the serogroup 078 . Binding of Congo Red is associated with presence of virulence genes such as *ompA* , *iss* and *fimH* and genes for multiple resistance to antibiotics (Catana et al., 2009 ; Fodor et al., 2010).

B. Hemolysis Test:

The results of hemolysis test indicated that 53.3% of pathogenic *E. coli* isolates were able to produce hemolysin (Fig 8.).
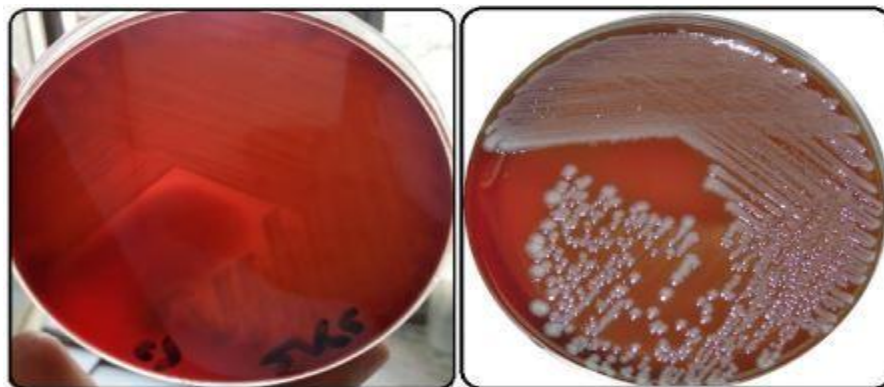
Fig.8: Hemolysis on blood agar

This result was in agreement with Fakruddin *et al.*, (2013) who isolated *E. coli* from non-enteric infections and found that (44.6 %) of clinical isolates 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 results of the present study were disagreed with that of Allan et al., (1993) who isolated 44 *E. coli* strains from cases of avian Colibacillosis and none of these tested strains appeared to produce hemolysin and with that of Rashid et al., (2013) who found that non of avian *E. coli* isolates were hemolytic .

C.Acriflavine test

The strain that isolated from clinical cases in Baghdad city showed variation in their agglutination strength.

Some strains showed negative result (no agglutination) and thus smooth strains are considered pathogenic . It has been shown that O antigen only present in strains displaying smooth-colony morphology , and strain displaying rough-colony morphology do not express O antigen and cannot be serotyped on the basis of O antigen (Poolman and Wacker , 2016) . Other strain that agglutinate showed variation in agglutination speed and size. The strains that agglutinate are rough strain and considered to be slightly pathogenic and cause mild clinical symptom. The rough strains agglutinate immediately at concentration (1:500) in less than half a minute. The results showed that 15 out of 17 (88.24 %) isolates were negative to acriflavine test i.e. pathogenic (Kloryga, 1986) .

Table 3 : Results of *in vitro* pathogenicity tests

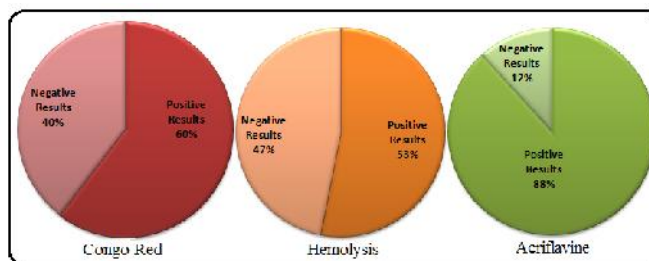| Pathogenicity Test	No. of Samples	Positive Results	Negative Results
Congo Red	53	32	21
Hemolysis	32	17	15
Acriflavine	17	15	2

Fig .9 : Results (Percentage) of *in vitro* pathogenicity tests

D. Serological Tests:

The 01,02 and 078 monovalent antisera which have been used in this study revealed that 7/15 tested isolates (47 %) were identified as *E.coli* serotype 02, 5/15(33 %) were identified as *E. coli* serotype 078 and 3/15(20%) were 01 serotype as shown in Fig.10 and Table 4:

Table 4 : Distribution of isolates among different serogroups

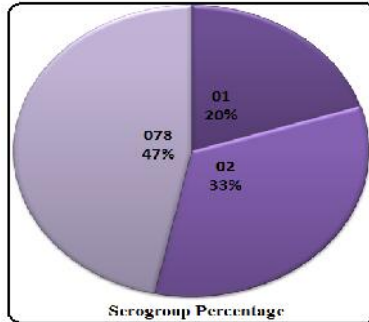| Number of Isolates	Serogroups		
	01	02	078
15	3	5	7

Fig 10 : Distribution of isolates (Percentage) among different serogroups

Schouler et al., (2012) stated that serotyping remains the most frequently used diagnostic method in laboratories, but it only allows the identification of a limited number of APEC strains. This method cannot therefore be used as an effective diagnostic tool, particularly since serotype does not reflect the virulence trait.

Kim and Namgoong, (1987); Allan et al., (1993) stated that the large percentage of strains was common characteristics of all groups of *E. coli* recovered from avian Colibacillosis regardless of geographic location.

E. Selection of *E. coli* strains that used in this study:

Among these serotypes, three isolates were chosen for further studies. Those were selected depending in vitro pathogenicity tests such hemolysin and Congo Red. Selected 01 isolated from blood, 02 heart fibrinousmaterial, and 078 from airsacfibrinous material.

In Vivo lethality testing:

A.One-day-old chick lethality test :

After inoculation, all groups were watched 24-96 hrs. to investigate: Mortality rate was 100% for 078 and 02 while 6/7(85.71%) for 01, control group all survive (Table 5 , Fig.11).

Table 5: Effect of *E. coli* serogroups on birds survival (In vivo lethality test at 1 day old).

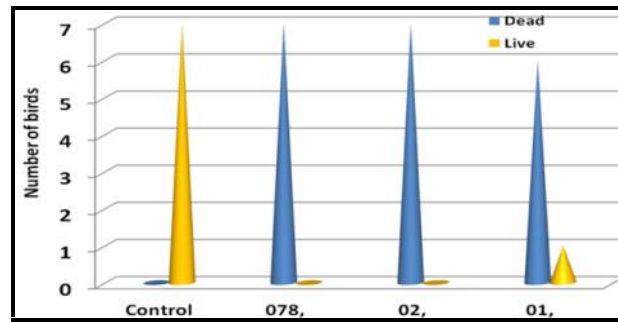| Groups	Dead Number	Live Number	Mortality Rate (%)
Control	0	7	0
078	7	0	100
02	7	0	100
01	6	1	86

Fig.11: Effect of experimental infection with *E. coli* serogroups on birds survival
(*In vivo* lethality test at 1 day old).

Post mortem lesion, all three treated groups exhibited generalized congestion, no signs appeared in control group as in Fig.12

Fig.12 Post mortem lesion of experimental infection with *E. coli* at one day old

E. coli was isolated from blood sample of most group. The isolated bacteria were checked for identification and serotyping.

APEC is airborne disease, so intranasal administration considered as the typical route for infection , and it would be more closely resemble to the natural route of infection (Smith et al., 1985 ; Matthijs et al., 2003) .

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) hrs. following S/C inoculation of bacteria . Mortality rate was 100% for 078 and 02 while 6/7(86 %) for 01, while the control group all survive.

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 85% mortality in

the one-day- old chicks following inoculation. These findings were in concordance with those of Ngleka et al., (2002) who recorded a variable degree of virulence ranging from high to moderate . Schouler et al.*,* (2012) were used one-day-old chicks lethality test as a model for pathogenicity testing of *E. coli* isolates

Fourteen day Old Chick Lethality Test :

Twenty eight broiler chickens were used for this test . Divided into 4 groups, 7 birds each. Group1 received 0.5ml of 10^8 CFU/ml (078) intranasal, group2 and group3 received similar dose in similar route for (02) and (01) respectively , group 4 was left as control. All groups were watched for 7day, to investigate:

Mortality rate: group1 (078) it was 5/7 (71.43), group2 the rate was 4/7 (57%) , group3 the rate was 4/7 (57%), group 4 (control) was unaffected (Table 6, Fig.13) .

Table 6: Effect of experimental infection with *E. coli* serogroups on birds survival (In vivo lethality test at 14 days old).

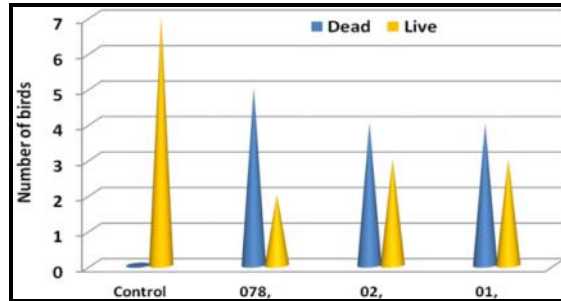| Groups	Dead Number	Live Number	Mortality Rate (%)
Control	0	7	0
O78	5	2	71.4
O2	4	3	57.1
O1	4	3	57.1

Fig 13: Effect of experimental infection with *E. coli* serogroups on birds survival (*In vivo* lethality test at 14 days old) .

Postmortemlesions exhibited sever to mild airsacculitis either one or double sided, fibrinous pericarditis, fibrinousperihepatitis and visceral congestion.

Fig.14: Experimental infection with *E. coli* at 14 days : Raffled feathers and depression (Left) , fibrinous pericarditis, fibrinousperihepatitis (Right)

It is clearly displayed characteristic pathological lesions and were in agreement with Dho-Moulin and Fairbrother (1999) recorded the same changes in experimentally and naturally infected broiler chickens with *E. coli*. On the seven day, the survival were killed and subjected for P.M examination. This examination revealed alleviated symptoms.

E. coli was isolated from blood sample of most groups. The isolated bacteria were checked for identification and serotyping as quantitative test (Smith et al., 1985).

Considering the results of in vivo tests, it seemed probable that this tests (One and fourteen -old chick lethality tests) are important for documentation the in vitro results as it clearly revealed the virulence characteristics of the studied isolates.

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