Prevalence of *E.Coli* in broiler chickens in winter and summer seasons by application of PCR with its antibiogram pattern.

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A B S T R A C T

A total of 205 chicken samples from apparently healthy broiler chickens (35 and 30), diseased broiler chickens (35 and 30) and freshly dead ones (35 and 40) were collected in winter (from December to February) and summer (from June to August) seasons, respectively from Menofyiea government. The results showed that the incidence of *E.coli* in apparently healthy broiler chickens was 15.7%, diseased broiler chickens 37.1% and in freshly dead ones 55% in winter season while in summer season was 15.8% in apparently healthy, 17.5% in diseased broiler chickens and 18.7% in freshly dead one. The serogroups of *E.coli* that obtained by serological identification were O_{128}, O_{78}, O_{111}, O_{124}, O_{55}, O_{142}, O_{114}, O_2 and O_1. The results of antibiotic sensitivity test for isolated *E.coli* showed that the isolated *E.coli* were highly sensitive for norfloxacin (60%), gentamycin(50%) , neomycin (50%) , streptomycin(50%) and chloramphenicol (50%). moderately sensitive for doxcyclin (10%) and erythromycin(40%) and highly resistant for amoxacillin /clavulinic acid (0%). The results of multiplex PCR showed that *eae A*(intimin or *E.coli* attaching and effacing) gene detected in O_{128},O_{55}, $O_1$and O_2, *OmpA* (*outer membrane protein*) gene detected in all *E.coli* serogroups that isolated , stx_1 gene not detected in all *E. coli* sergroups that isolated ,but stx_2 gene detected in O_{114} and O_{128}.

Keywords: *E.coli*, Broiler chickens, Seasons, PCR, Antibiogram pattern.

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1. INTRODUCTION

E.coli typically colonizes the gastrointestinal tract of warm-blooded animals within a few hours after birth. However, a large number of highly adapted *E.coli* pathogens have acquired specific virulence attributes (kaper et al., 2004). Some pathotypes of *E.coli* are capable of causing intestinal diseases, while others referred to as extraintestinal pathogenic *E.coli* (ExPEC), are responsible for extraintestinal infections. Avian pathogenic *E.coli* (APEC), fall under the category of ExPEC (Mellata, 2013) that induces different syndromes in poultry including, systemic and localized infections such as respiratory colibacillosis, acute colisepticemia, salpingitis, yolk sac infection, and swollen-head syndrome (Dho-Moulin and Fairbrother, 1999). Colibacillosis is a widespread disease, which is responsible for severe economic losses for the world's poultry industries. The most common form of colibacillosis is characterized by an initial respiratory disease, which is usually followed by a systemic infection with characteristic fibrinous lesions (airsacculitis, perihepatitis and pericarditis) and fatal septicemia. The infection is generally initiated or enhanced by predisposing agents, such as mycoplasmal, viral infections and environmental factors (Dho-Moulin and

Fairbrother, 1999); (Barnes et al., 2008). *E.coli* can survive in dry, dusty conditions for long periods and it has been shown that wetting the litter can reduce the incidence of colisepticaemia (Black, 1990), probably due to a reduction in the numbers of *E.coli*. Feed ingredients and water are often contaminated with pathogenic coliform and are common source of introducing new serotypes into a flock (Martins, et al., 2007). The species of E.*coli* are serologically divided in serogroups and serotypes on basis of their antigenic composition (somatic or O antigens for serogroups and flagella or H antigens for serotypes).Many strains express a third class of antigens(capsular or K antigens) (Compos et al., 2004). Antimicrobial therapy is an important tool in reducing both the incidence and mortality associated with avian colibacillosis. The long-term use of antimicrobials for therapy and growth promotion in poultry resulted in drug resistance in Gram-negative pathogens (Singer and Hofacre, 2006). Serogrouping and detection of some virulence associated genes in randomly selected isolates using a previously designed multiplex PCR (Johnson et al., 2008). This study aimed to determine the prevalence, serotypes and antimicrobials susceptibility profile of avian pathogenic *E.coli* (APEC) strains in broilers farms in winter and summer seasons in Menofya Government, Egypt and detection of some virulence genes of the isolated strains by using PCR.

2. MATERIAL AND METHODS

2.1. Chicken samples

A total of 205 chicken samples from apparently healthy broiler chickens (35 and 30), diseased broiler chickens (35 and 30) and freshly dead ones (35 and 40) were collected in winter (from December to February) and summer (from June to August) seasons, respectively from Menofya government. The samples were collected from liver, Heart blood, kidneys and spleen.

2.2. Detection of E.coli by conventional method: it was done according to Quinn et al. (2002)

2.2.1. Selective enrichment of E.coli

Each sample was inoculated separately into buffer peptone water and incubated at 37˚C for 18 -24 hrs under aerobic condition.

2.2.2. Colonization of E.coli on selective differential solid media

A loopful from the broth of each sample was streaked onto MacConkey's agar and Eosin Methylene blue agar. The inoculated plates were incubated at 37°C for 24 hours. Suspected *E.coli* colonies were purified and kept for further identification.

2.2.3. Identification of suspected E.coli colonies

It was performed according to Quinn et al., (2002): On MacConkey's agar and Eosin Methylene blue agar (EMB).

2.2.4. Microscopic examination

Gram's stain was prepared and used as described by (Cruickshank et al. 1975) for morphological study.

2.2.5. Biochemical Identification

According to Quinn et al. (2002) including Indole reaction, Methyl red test, Voges Proskauer test, Citrate utilization test, Catales test, Sugar fermentation test, Oxidase test, Triple sugar iron and Christener's urea agar test.

2.2.6. Serological identification of E.coli (Edwards and Ewing (1972)

Isolated strains were serotyped in animal health research institute, Dokki, Giza using: Polyvalent and monovalent diagnostic *E.coli* antisera.

2.3.Antibacterial sensitivity test

The disk diffusion technique was applied according to (Cruickshank et al., 1975). Eight antibiotic discs were used including

Table (1) Incidence of *E. coli* infection in winter and summer seasons in chicken samples

Sample	Winter			Summer			Total		
	No. of sample	No. of +ve	%	No. of sample	No. of +ve	%	No. of sample	No. of +ve	%
Apparently healthy	35	13	37.1	30	10	33.3	65	23	35.3
Diseased	35	19	54.2	30	12	40	65	31	47.6
Freshly dead	35	32	91.4	40	19	47.5	75	51	68
Total	105	64	60.9	100	41	41	205	105	51.1

% was calculated according to the numbers of examined broiler chickens.

Table (2): Incidence of *E.coli* infection in different organs in winter season:

Sample	liver N =105		Heart blood N =105		Spleen N =105		Kidney N =105		total N =420	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
Apparently healthy N =35	3	2.8	6	17.1	7	20	6	17.1	22	15.7
Diseased N =35	16	45.7	11	31.4	10	28.5	15	42.8	52	37.1
Freshly dead N =35	22	62.8	21	60	20	57.1	14	40	77	55
Total N =105	41	39.04	38	36.1	37	35.2	35	33.3	151	35.9

% was calculated according to the numbers of examined broiler chickens.

amoxacillin/claviulinic, chloramphenicol, erythromycin, doxycyclin, streptomycin, gentamycin, neomycin and norfloxacin). The interpretation of inhibition zones of tested culture was according to CLSI, (2012).

2.4. Virulence genes of E.coli detection by PCR

Multiplex PCR was applied by using four sets of primers for detection of four virulence genes that may play a role in virulence of APEC.

These genes were *eaeA* (intimin or *E.coli* attaching and effacing gene); *ompA* (outer membrane protein); *stx1* (shiga-toxin1 gene) and *stx2* (shiga-toxin2 gene). It was applied on isolated *E.coli* following QIA amp DNA mini kit instructions (Catalogue no.51304); Emerald AmpGT PCR master mix (Takara) Code No.RR310A kit and agarose gel electrophoreses by Sambrook et al., (1989).

2.5.Antibacterial sensitivity test

The disk diffusion technique was applied according to (Cruickshank et al., 1975). Eight antibiotic discs were used including amoxacillin/claviulinic, chloramphenicol, erythromycin, doxycyclin, streptomycin, gentamycin, neomycin and norfloxacin). The interpretation of inhibition zones of tested culture was according to CLSI, (2012)

3. RESULTS

3.1. Incidence of E.coli infection in broiler chicken samples in winter and summer seasons

Morphologically *E.coli* isolates were gram - ve rods appeared as pink colonies when cultured on MacConkey media and green

metallic colonies on EMB medium. Biochemically, all *E.coli* suspected isolates were lactose fermenting colonies, positive indole, methyl red, and Catalase. Meanwhile all isolates were negative oxidase, urea hydrolysis, citrate utilization, Voges-Proskauer and didn't produce H_2S.

The prevalence of suspected *E.coli* isolates from dead chickens was 55%, followed by diseased broiler chickens was 37.1% and from apparently healthy broiler chickens was 15.7% in winter and isolated from dead chickens 18.7%, followed by diseased broiler chickens was 17.5% and from apparently healthy broiler chickens was 15.8% in summer season.

Table (3): Incidence of *E.coli* infection in different organs in summer season:

Sample	liver N =100		Heart blood N =100		Spleen N =100		Kidney N =100		total N =400	
	+ve	%	+ve	%	+ve	%	+ve	%	+ve	%
Apparently healthy N =30	7	20	6	23.3	3	10	3	10	19	15.8
Diseased N =30	9	26.6	8	30	3	10	1	3.3	21	17.5
Freshly dead N =40	11	27.5	11	27.5	3	7.5	5	12.5	30	18.7
Total N =100	27	27	25	25	9	9	9	9	70	17.5

% was calculated according to the numbers of examined broiler chickens.

This indicates that the prevalence of *E.coli* isolates is higher in winter than summer table (1).

3.2. Recovery rate of E.coli from internal organs

The high incidence of *E.coli* was recovered from liver 39.04% and 27%, followed by fresh heart blood 36.2% and 25% , spleen 35.2% and 9% and kidneys 33.3% and 9% both in winter and summer seasons ,respectively (table 2 and 3).

3.3. Serotyping of E.coli isolates isolated from examined broiler chickens samples

The most commonly detected E.coli serogroups were O128, O78, O111,O114, O55, O124, O142, O1 and O2 (table, 4).

Table (4) Serotyping of *E.coli* isolates recovered from chicken sample

Isolated serogroups	No of isolate	%
O128:H2	5	23.8
O78	4	19.04
O111:H4	3	14.28
O124	2	9.5
O55:H7	2	9.5
O142	1	4.7
O2:H6	2	9.5
O114	1	4.7
O1:H7	1	4.7

% were calculated according to the numbers of examined broiler chickens

3.4. Antibiotic sensitivity test of the isolated E.coli strains

By using different eight antibiotic discs we found that the isolated *E.coli* were highly sensitive for norfloxacin 60%, gentamycin 50%, neomycin 50%, streptomycin 50% and chloamphenicol 50% but they were moderately sensitive for doxcyclin 10% and erythromycin 40% and highly resistant for amoxacillin /clavulinic acid (table, 5).

Table (5) Result of antibiotics resistance of *E.coli* by disc diffusion method

Isolate	A	S	E	C	DO	NOR	G	N
S	0	5	4	5	1	6	5	5
I	0	0	3	2	2	1	2	2
R	10	5	3	3	7	3	3	3
% *	0	50	40	50	10	60	50	50

Sensitivity percent, G: gentamycin, E: erythromycin, S: streptomycin, C: chloramphenicol, A: Amoxacillin /clavulinic acid, DO:Doxycyclin, NOR:Norfloxacin, N:Neomycin

3.5. PCR for Detection of some virulence Genes of E.coli

The results of multiplex PCR showed that *eae A* gene detected in O_{128} ,O_{55} ,O_1 and O_2, *OmpA* gene detected in all *E. coli* serogroups that isolated O_{78},O_{111}, O_{128}, O_{55}, O_2,O_1 ,O_{142},$O_{114}$and O_{124} , *stx*$_1$ gene not detected in all *E.coli* sergroups that isolated ,but *stx*$_2$gene detected in O_{114} and O_{128} (Table ,6) (Figure 1,2,3,4).

Table (6) the results of PCR amplifications of different used genes of *E. coli* serogroups

Sample	eaeA	ompA	stx1	Stx2
1(O78)	-	+	-	-
2(O111:H4)	-	+	-	-
3(O114:H2)	-	+	-	+
4(O128:H2)	+	+	-	+
5(O55:H7)	+	+	-	-
6(O2:H6)	+	+	-	-
7(O142)	+	+	-	-
8(O124)	-	+	-	-

eaeA (intimin or E.coli attaching and effacing gene), ompA(outer membrane protein).stx2(shiga-toxin2 gene), stx1(shiga-toxin1 gene)

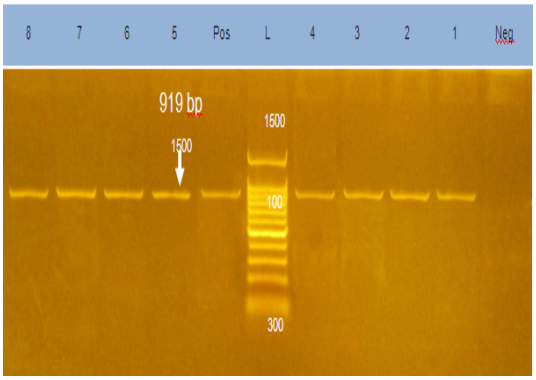

Figure (1): Results of PCR for amplification of *ompA* gene of *E.coli* serogroups, Lane L: 100-1500bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1, 2, 3, 8: *E.coli* O_{78} & O_{111}:H_4& O_{114}:H_2&O_{124} (positive). Lane4, 5 : *E.coli* O_{128}: H_2&O_{55}: H_7 (Positive). Lane 6,7 : *E.coli* O_2: H_6& O_{142} (Positive)

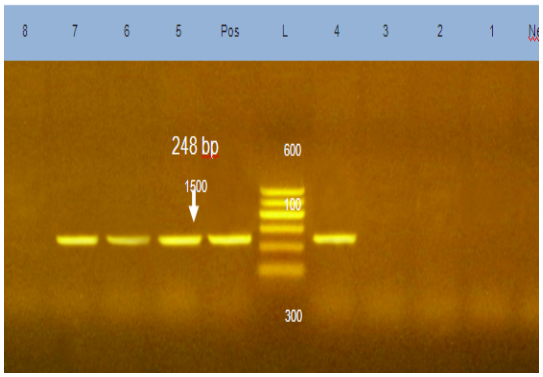

Figure (2): Results of PCR for amplification of *eaeA* gene of *E.coli* serogroups. Lane L: 100-600bp DNA Ladder .Neg. Negative control.Pos. : Positive control.Lane 1, 2, 3, 8: *E.coli* O_{78} & O_{111}: H_4 & O_{114}: H_2 & O_{124} (Negative). Lane 4, 5:O_{128}: H_2 & O_{55}:H_7 (Positive). Lane 6, 7: O_2: H_6 & O_{142} (Positive)

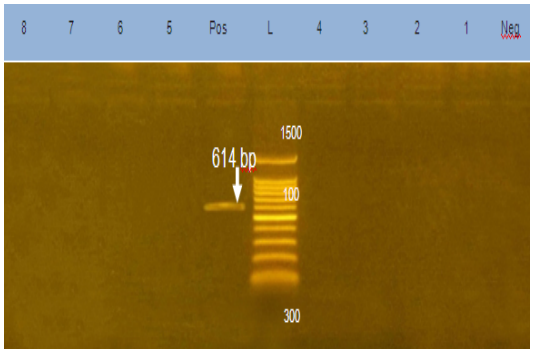

Figure (3): Results of PCR for amplification of *stx*$_1$ gene of *E.coli* serogroups: Lane L: 100-1500bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1, 2, 3, 8: *E.coli* O_{78} & O_{111}: H_4 & O_{114}: H_2& O_{124} (Negative). Lane 4, 5: *E.coli* O_{128}: H_2 & O_{55}: H_7 (Negative). Lane 6, 7*: E.coli* O_2: H_6& O_{142} (Negative)

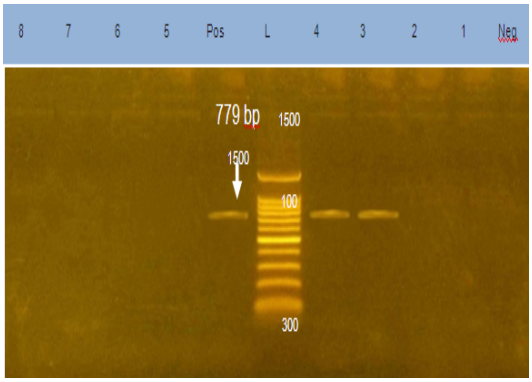

Figure (4): Results of PCR for amplification of *stx*$_2$gene of *E.coli* serogroups .Lane L: 100-1500bp DNA Ladder. Neg.: Negative control. Pos.: Positive control. Lane 1, 2, 5, 8: *E.coli* O_{78} & O_{111}: H_4 & O_{55}: H_7 & O_{124} (Negative). Lane4, 3: *E.coli* O_{128}: H_2 & O_{114}: H_2 (Positive). Lane 6, 7: *E.coli* O_2: H_6 & O_{142} (Negative)

4. DISCUSSION

E.coli is considered a member of the normal microflora of the poultry intestine but certain strains such as those designated as avian pathogenic *E.coli* (APEC) spread into various internal organs and cause colibacillosis characterized by systematic fatal disease (Someya et al ., 2007).Typing of isolated bacteria including *E.coli* could be achieved by phenotypic and/or genotypic protocols. The phenotypic characteristic method used for identification of *E.coli* includes the morphological and biochemical tests. Most of these techniques are not sufficiently sensitive to distinguish between different strains and they are affected by physiological factors (Fantasia et al., 1990). Therefore, serological protocol was established to differentiate *E.coli* isolates. Regarding the morphological characters used for identification of E.coli, depend on that *E.coli* isolates are Gram-negative rods with pink colonies when cultured on MacConkey agar media, green metallic colonies on EMB medium. Nearly similar results were noted by Kumar et al., (1996) and Hogan and larry (2003).

Bacteriological study was conducted on 820 randomly collected organ samples from apparently healthy broiler chickens, diseased broiler chickens and freshly dead ones including liver, fresh heart blood, kidneys and spleen isolated from four broiler farms located in Menofyiea government in winter and summer seasons revealed that *E.coli* isolates was recovered from 221 samples with overall prevalence 27.3%,This study revealed that the *E.coli* isolates were isolated from 26.9% (221 out of 820) broiler chickens samples originated from different sources including; Fresh heart blood 30.7% (63out of 205) Liver 33.1% (68 out of 205), Kidneys 21.5% (44 out of 205) and Spleen 22.4% (46 out of 205) . These results are agreed to some extend with that obtained by Abd El Tawab ,(2014) who isolated *E.coli* at a percentage of 38%. From the above mentioned results, it is obvious that *E.coli* isolates were recovered from poultry farms with higher prevalence from liver samples followed by Fresh heart blood, spleen and kidneys. Nearly similar result obtained by El Sayed et al., (2015).

The incidence of *E.coli* among examined chickens in winter was 60.9% and this percentage was higher than that in summer 41% .This variation may be attributed to defects in the environmental and hygienic condition in poultry farms in winter as bad ventilation, overcrowding and high amount of ammonia in air also may be due to high incidence of *E.coli* in water, feed, litter and air in winter than in summer .These results agreed with those obtained by Nehal, (2009), Mahajan et al., (1994) and Ayoub ,(2007) .

It was observed that several serotypes were recovered from clinical cases of broiler chickens with different *E.coli* infection as O_{128} ,O_{78},O_{111} , O_{124}, O_{55}, O_{114} ,O_{142}, O_2 and O_1 (Table,4). Similarly *E.coli* serotypes had been previously isolated from chicken and newly hatched chicks in Egypt as reported by Abd El-Haleem ,(2000) were O_{78} and O_{111} , Taha et al., (2002) was O_2, El-Sayed et al., (2015) were O_{111},O_{55},$O_{142}$and O_{128} and Reem ,(2015) were O_{142},O_1, O_{55},$O_{128}O_{114}$ and O_{124}, respectively.

The results of antibiotic sensitivity tests (Table, 5) revealed that gentamycin, doxycyclin, norfloxacin and chloramphenicol were the most proper antibiotics with the highest in vitro efficiency against the isolated *E.coli*. These results go in parallel with those obtained by Nehal, (2009), Sharada et al., (2010), Tapanet al., (2012) and Abd El Tawab, (2014). Results of antimicrobials sensitivity of serotyped *E.coli* recovered from broilers showed that the majority of *E.coli* isolates were sensitive to gentamycin (60%), norfloxacin (60%), streptomycin (50%), neomycin (50%) and chloramphenicol (50%).The results were nearly similar to that obtained by Sharada et al., (2010).

The results of antibiogram in this study are in variance with the findings of other workers, indicating that antibiotic pattern varies with different isolates, time and development of multiple drug resistance among different *E.coli* isolates related to transmissible R factor /plasmid. The resistance plasmid from *E.coli* has been transmitted from poultry to human (Tapanet al., 2012).

The results revealed that all *E.coli*isolates recovered from various chicken broiler samples were negative for *stx*$_1$ in *E.coli* isolates (Table, 6) and (Figure, 3) .Nearly similar findings were recorded by Ahmed (2011), Mona et al., (2013) and Homaira et al., (2015). The results of PCR amplification of *Stx*$_2$gene in isolated *E.coli*strains showed that out of 8 *E.coli*isolates, one (O128) was positive forthe *Stx*$_2$gene yielded a consistent fragment of 779 bp. (Table, 6) and (Figure, 4). These results substantiate what has beenreported by Abd El Tawab, (2014). Concerning the examination of *E.coli*isolates for the detection of *intimin(eaeA)* gene demonstrated that four isolates (O_{128} , O_{55}, O_1 and O_2)out of eight isolates, yielded the expected size of 248 bp PCR amplification products for the *intimin*gene (Table,6)and **(**figure,2). These findings were nearly agreed with those obtainedby Ahmed et al., (2007) and Ahmed Al-Ajmi (2011). Finally, PCR amplification of *ompA*gene in isolated *E.coli* strains showed that the *ompA* gene was amplified in all *E.coli*serogroups that were isolated giving a PCR product of 919bp (Table 6) and (Figure 1). Similar findings were recorded by Catanaet al., (2008), Johsonet al., (2008), and Zhao et al., (2009) who reported that *ompA* gene was found in all APEC isolates.

Conclusion: *E.coli* could be isolated from examined samples in different farms under investigation in either winter or summer seasons. Also the isolation rate was higher in winter than in summer season and detection of some virulence genes from isolated serogroups by application of PCR.

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