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Presence of Non – O157 Shiga Toxin Producing *Escherichia coli* in Broiler chickens

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ABSTRACT

Shiga toxin (Stx)-producing *E. coli* (STEC) is one of the most important pathogens that infected broiler chickens and cause large economic loses in Egypt broiler farms. The present study was designed to determine the prevalence and identification of Non – O157 Shiga toxin (Stx) -producing *E. coli* in broiler chickens as a hazard in broiler farms. For that a total of 200 cases of broiler chicken samples (lung, liver, heart and gall bladder) were collected from100 freshly dead birds and 100 diseased birds suffering from chronic respiratory disease (CRD) or colibacillosis, from 10 broiler farms showing high mortality ratesin Bohera Governorate, and analyzed in bacteriology laboratory by cultured in some different bacteriological media followed by gram's staining, biochemical tests, serological tests and Polymerase Chain reaction (PCR). The PCR was performed by targeting Shiga toxin producing genes (Stx), (Stx1 and Stx2) in *E. coli*. *E. coli* was isolated from 40 (40%) cases in diseased birds, 66 (66%) cases in freshly dead birds, and 106 (53%) cases in both diseased and freshly dead birds. The *E. coli* prevalence was higher in freshly dead broiler chickens than diseased broiler chickens. In both diseased and freshly dead broiler chickens, the *E. coli* prevalence was higher in lung samples 41/200 (20.5%), followed by liver samples 30/200 (15%), then heart samples 20/200 (10%), and the gall bladder samples were the lowest prevalence15/200 (7.5%). Serodiagnosis of 10 random selected *E. coli* strains resulted in ; 2 serotypes O119: H6, 1 serotypeO1:H7, 3 serotypes O146:H21 and 4 serotypes O78. These 10 serotyped strains were further detected by PCR for the (Stx) genes and revealed that, only one strain O146:H21 was positive for the(Stx1)gene, and two strains O78 were positive for the (Stx2)gene. Our current study concluded that, broiler chickens may act as a source of Shiga toxin-producing *E. coli* (STEC) infection (Stx1 and Stx2), which may leading to mortalities in broiler chickens and cause large economic loses.

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INTRODUCTION:

Escherichia coli is one of the most common pathogens responsible for colibacillosis in broiler chickens leading to cost-effective losses among poultry production around the world, as it causes poor performance and high death rates. Avian pathogenic *E. coli* causes chronic respiratory illness, omphalitis, synovitis, coligranulomatosis and salpingitis. Colibacillosis is diagnosed primarily through clinical symptoms, polyserocytis lesions, and pathogen isolation and identification. Colibacillosis is a major cause of death in broiler chickens of all ages.

Although chickens of all ages are susceptible, younger chickens have a more severe type of sickness than older chickens **(Waffa and Aqeel. 2023)**. E.coli isolated from different breeding stages of a broiler chicken flocks, and it was the most prevalent subtype in the broiler chicken farms and persisted throughout layer breeding **(Liao et al. 2023).** A recent study **(Pais et al. 2023*)*** revealed that *E. coli* causes a major risk on the broiler chickens which leading to large economic losses in broiler industry. Avian pathogenic *E. coli* cause serious disease in broiler chickens, which mainly characterized by perihepatitis, airsaculitis and pericarditis resulting in large economic losses in broiler industry worldwide**.**

E. coli is harmful bacteria that consists of several strains as enterohaemorrahgic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), Shiga toxin-secreting (STEC), diarrhea-associated hemolytic (DHEC), enter aggregative (EAAggEC) and cytolethal distending toxin recreating (CDTEC) that have side effects on the health, infections with (Stx1) and (Stx2) producing *E. coli* leads to hemorrhagic colitis **(Butler. 2012).**

Elsayed et al. (2021) reported that STEC isolates were confirmed in broiler chickens, and revealed that the Shiga toxin-producing *E. coli* (STEC) represented a great risk to public health**.**

Sokolovic et al. (2022) revealed that Shiga-toxin producing *E. coli* produces one or more toxins that are known as Shiga toxins. *E. coli* Shiga toxins (Stx) constitute a family of several related cytotoxins, at least two of them, (Stx1) and (Stx2), which are encoded by (Stx1) and (Stx2)genes, respectively ***(*Kiandokht et al. 2021).**

Ray and Singh. (2022) also concluded that the main virulence factor of STEC is the Shiga toxin (Stx) genes encoded on temperate bacteriophages (Stx phages). The major virulence factors involved in STEC infection are the powerful Shiga toxins, which are classified into 2 main groups: (Stx1) and (Stx2). Shiga toxin 2 (Stx2) reported as one of the most powerful toxin, and the toxin producing strains are usually associated with more severe infections and illness **(Gyles. 2007).**

Femi et al. (2021) said that Shiga toxins bind to the surface of eukaryotic cells, inhibit protein synthesis, thereby causing the death of their hosts as broiler chickens. they also reported that Shiga toxin is the major factor invirulence of *E. coli* and there are two toxin forms, (Stx1) and (Stx2) encoded by (Stx1) and (Stx2) genes.. also, **Brito et al. 2003** have reported that *E. coli* isolated from chickens may harbor Shiga toxin-encoding genes .

Non – 157 Shiga toxin–producing *E. coli* (STEC) are emerging pathogens **(Scallan et al. 2011)**, but are under recognized because relatively few clinical laboratories routinely use culture-independent testing methods necessary for their prevalence and identification **(Kaspar et al. 2011).**

This study was adopted on analysis and detection of Non – 157 Shiga toxin (Stx)-producing *E. coli* (Stx1 and Stx2) in broiler chickens due to their hazard, which may leading to mortalities in broiler chickens and cause large economic loses.

MATERIALS and METHODS

Collection of samples& Transportation:

A total of 200 cases of broiler chicken samples (lung, liver, heart and gall bladder) were collected from 100 freshly dead birds and 100 diseased birds suffering from chronic respirato-

ry disease (CRD) or colibacillosis, from 10 broiler farms showing high mortality rates in Bohera Governorate, as shown in table (1), and kept in ice box then immediately transported to the laboratory.

Table 1. Number of broiler chicken cases:

Broiler chicken cases	Samples	
	No.	%
Diseased	100	50
Freshly dead	100	50
Total	200	100

No= number of broiler chicken cases. %= percent of broiler chicken cases.

Isolation and Identification of *E. coli*:

Tissue samples were inoculated onto Mac-Conkey agar and incubated for 24 hrs at 37ºC. Lactose fermenting colonies were subcultured onto Eosin Methylene blue agar and blood agar and incubated at 37ºC for 24 hours according to **Quinn et al. (2002).** Suspected E. coli colonies with metallic sheen were subjected to biochemical tests.

Biochemical identification:

Suspected *E. coli* colonies were tested biochemically by applying the following tests: (Oxidase, Catalase, Urease, Methyl Red, Vogues Proskaur, Citrate utilization, Nitrate reduction, Indole and TSI) according to **Kreig et al. (1984).**

Serotyping of *E. coli:*

10 random selected isolates proved biochemically *as E.coli* were subjected to serological identification by using rapid diagnostic *E. coli* antisera sets **(DENKA SEIKEN Co., Japan)** according to **Kok et al. (1996)**.

Detection of the (Stx1) and (Stx2) genes by using PCR:

DNA extraction:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56ºC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primers:

Primers used were supplied from **Metabion (Germany),** which are listed in table (2).

PCR amplification:

Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 2. Target genes, primers sequences, amplicon sizes and cycling conditions (Dipineto et al. 2006):

Target gene	Primers sequences	Amplified segment (bp)	Primer Den.	Amplification (35 cycles)			Final extension
				Sec. den.	Ann.	Ext.	
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCAC-TTTG	779	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.

Stx1=Shiga toxin 1, Stx2=Shiga toxin 2, Den., denaturation, Sec. den., secondary denaturation, Ann., annealing & Ext., extension.

RESULTS

Among 200 cases of broiler chickens (100diseased and 100 freshly dead) tested bacteriologic ally, *E. coli* was isolated from 40 (40%) cases in diseased birds, 66 (66%) cases in freshly dead birds, and 106 (53%) cases in both diseased and freshly dead birds, as shown in table (3).

Table 3. Incidence of detected *E. coli* in diseased and freshly dead broiler chicken cases:

Broiler chicken cases	Broiler chickens No.	*E. coli* isolates	
		No.	%
Diseased	100	40	40
Freshly dead	100	66	66
Total	200	106	53

No.= number of broiler chickens samples. %= percent of broiler chickens samples.

Among 200 cases of broiler chickens (100 diseasedand100 freshly dead) samples (lung, liver, heart and gall bladder), the prevalence of *E. coli* was recorded with a percent of 20.5% (41/200), 15% (30/200), 10% (20/200) and 7.5% (15/200) from lung, liver, heart and gall bladder, respectively, as shown in table (4).

Table 4. Incidence of detected *E. coli* in organs of both diseased and freshly dead broiler chickens:

Broiler chicken cases	Broiler chickens No.	*E. coli* isolates from organs								Total isolates	
		Lung		Liver		Heart		Gall bladder			
		No.	%	No.	%	No.	%	No.	%	No.	%
Diseased	100	15	15	12	12	8	8	5	5	40	40
Freshly dead	100	26	26	18	18	12	12	10	10	66	66
Total	200	41	20.5	30	15	20	10	15	7.5	106	53

No.= number of *E. coli* isolates. %= percent of *E. coli* isolates.

Serodiagnosis of 10 random selected *E. coli* strains revealed that: 2 serotypes O119:H6, 1 serotypeO1:H7,3 serotypes O146:H21 and 4 serotypes O78, as shown in table (5).

Table 5. Serodiagnosis of 10 *E. coli* strains:

E. coli serotype	Number
O119:H6	2
O1:H7	1
O146:H21	3
O78	4
Total	10

The 10 serotyped strains were further detected by PCR for Shiga toxin genes (Stx1 and Stx2), as shown in table (6), and resulted in only one strain (O146:H21) was positive for the Shiga toxin 1 (Stx1)gene with a percent of 10%, as shown in figure (1), and two strains (O78) were positive for the Shiga toxin 2 (Stx2)gene with a percent of 20%, as shown in figure (2).

Table 6. Incidence of virulence genes of Shiga toxin-producing *E. coli* (STEC), (Stx1 & Stx2) in 10 *E. coli* serotypes in Broiler chickens:

E. coli NO.	Serodiagnosis	Stx1	Stx2
1	O119: H6	-	-
2	O78	-	-
3	O1: H7	-	-
4	O146: H21	-	-
5	O146: H21	+	-
6	O78	-	-
7	O78	-	+
8	O119: H6	-	-
9	O78	-	+
10	O146: H21	-	-

Stx1=Shiga toxin 1, Stx2= Shiga toxin 2. += Positive. -= Negative.

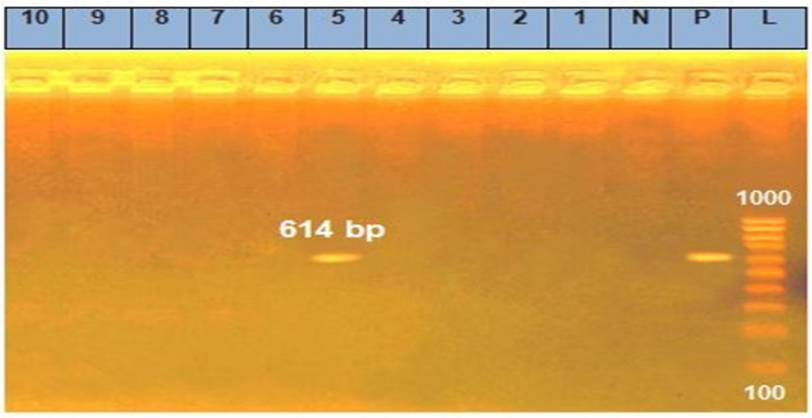

Figure (1): Agarose gel electrophoresis patterns showing PCR amplification products forStx1gene of *E.coli* serogroups.
Lane L: 100-1000bp DNA Ladder.
Lane P: Positive control.
Lane N: Negative control.
Lane 1, 2, 3, 4, 6, 7, 8, 9 & 10: *E. coli* O119:H6, O78, O1:H7, O146:H21, O78, O78, O119:H6, O78&O146:H21(Negative).
Lane 5: O146:H21(Positive).

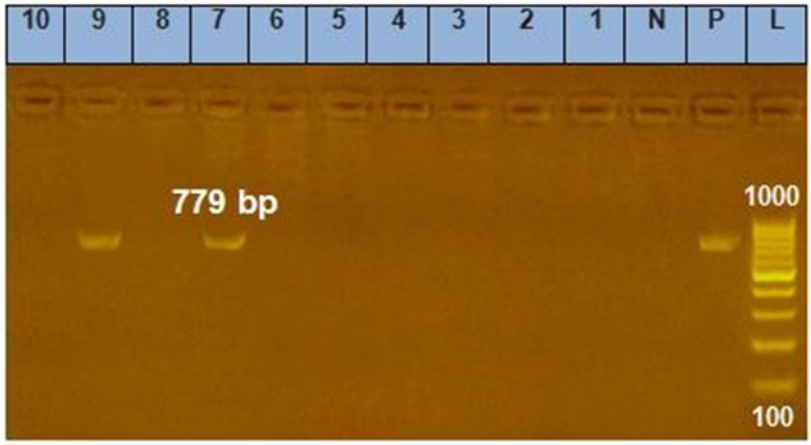

Figure (2): Agarose gel electrophoresis patterns showing PCR amplification products for Stx 2 gene of *E. coli* serogroups.
Lane L: 100-1000bp DNA Ladder.
Lane P: Positive control.
Lane N: Negative control.
Lane 1, 2, 3, 4, 5, 6, 8 & 10*: E. coli* O119:H6, O78, O1:H7, O146:H21,O146:H21, O78, O119:H6 &O146:H21(Negative).
Lane 7 & 9: O78& O78 (Positive).

DISCUSSION

E. coli is recognized as a common inhabitant of the broiler chickens intestinal tract which frequently causes contamination and infection and recorded among clinically important pathogens in broiler chickens **(Hossain et al. 2023)**. E. coli is considered a member of the normal microflora of the broiler chickens intestine but certain strains designated as avian pathogenic *E. coli* (APEC) spread into various internal organs and cause colibacillosis which characterized by systematic fatal disease in broiler chickens **(Someya et al. 2007).** E. *coli* infections in broiler chickens cause many clinical manifestations which characterized by a respiratory disease that is frequently followed by a generalized infection which end by death, avian pathogenic *E. coli* (APEC) strains fall under the category of extra intestinal pathogenic *E. coli*, which are characterized by the possession of virulence factors that enable to live extra intestinal life **(Johnson et al. 2006).**The STEC strains such as Non – O157E. coli have acquired genetic traits that made them potential risk **(Luna-Gierke et al. 2014)**.

In the current study E. *coli* isolated from broiler chickens with a percent of 53% which was nearly similar to published results of **(Adzitey et al. 2012)** which confirmed isolated *E. coli* from 50% of the broilers. Higher rates were recorded by **(Abd El Tawab et al. 2015** and **Hamza et al. 2016)** who recovered E. coli in 75% and 60% of the tested broiler chicken samples respectively. A lower rate was recorded by **(Hossain et al. 2023** and **Omid et al. 2021)** who isolated E. coli with the percentages of 31.25% and 38%, respectively.

Our study revealed that the prevalence of *E. coli* was higher in the freshly dead broiler chickens (33%), than diseased broiler chickens (20%). also, the prevalence of isolation of *E. coli* was higher in lung samples with a percent of 20.5%, followed by liver samples (15%), then heart samples (10%), and the gall bladder samples were the lowest prevalence (7.5%). these results were disagreed with **(Eid and Erfan 2013)** who reported the E. coli isolation rates from different organs with pathologic conditions, the maximum rate was recorded in liver showing perihepatitis 60 (57.14%), followed by lung showing pneumonia 57 (54.29%) and the least percentage was recorded from heart (pericarditis) 39 (37.14%) out of

the examined 105organs from each type (liver, lung and heart).

depend on the morphological characters used for identification of *E. coli*, *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. 2003)** and **(Hogan and larry. 2003)**. Serological test was established to differentiate *E. coli* isolates.

In our current study, serotyping of 10 random selected *E. coli* strains resulted in;2 serotypes O119:H6, 1 serotypes O1:H7, 3 serotypes O146:H21 and 4 serotypes O78, which were nearly similar to results obtained by **(Hamza et al. 2016)** study, who found the O119, O1 and O78 serotypes in broiler chickens, Also our results agreed with **(Hemmat et al. 2019)**who recorded O119.on the other hand our results disagreed with **(Eid and Erfan 2013)** who not reported any serotype similar to our study where recorded O145:K, O25:K11, O44:K74, O126:K71 and O118:K.

This study revealed that among O78 was the most prevalent which detected in 4/10 (40%) and which agreed with **(Elsayed et al. 2021)** that detected O78 E. coli most prevalent also in 6/60(10%), and agreed with **(Abd El Tawab et al. 2016)** who confirmed the high prevalence of O78 E. coli among strains that cause avian colibacillosis, also the higher prevalence of O78 *E. coli* serotype was in agreement with previous study of **(Dimitrios et al. 2022)** confirming the predominance of O78 *E. coli* in many parts of the world and in broiler chickens with coli septicemia. **Mishra et al. (2002)** found that the most predominant serotype reported was O78 *E. coli* among the *E. coli* strains isolated from 250 clinical broiler samples and that similar to we reported in our study.

In this study, O146:H21 was a higher percent 3/10 (30%) and that was agreed with **(Abd El-Mongy et al. 2018).** Also, (O1:H7) was among the serotyped *E. coli* strains detected in this study and that was agreed with **(Ewing and Ewing. 1986)**.

The serotyped E. coli strains were furthermore detected by PCR for Shiga toxin(Stx) genes and resulted in; only one strain O146:H21 was positive for theShiga toxin 1 (Stx1)gene with a percent of 10%, and two strains O78 were positive for the Shiga toxin 2 (Stx2)gene with a percent of 20%.

Our reported (Stx) gene sequences; one isolate carried (Stx1) sequence, two carried (Stx2) sequences, the two (Stx2) isolates were O78 and that agreed with **(Elafify et al. 2016)**. **Omid et al. (2021)** revealed that the E. coli virulence genes, (Stx1) and (Stx2) existed in pathogenic *E. coli* strains isolated from broiler and that the (Stx2) gene was the most frequent virulence factor than (Stx1) among the STEC isolates which agreed with our study. In our study, STEC was low and this was agreed with previous authors who confirmed with our results such as **(Farooq et al. 2009 and Ghanbarpour et al. 2011).**

Elsayed et al. (2021) found that The Shiga toxin gene(Stx1) was detected in all strains, while(Stx2) was detected in 80% of strains; the increased detection rate of (Stx1) and (Stx2) in broilers was disagreed with our study which revealed only one strain of (Stx1) and two strains of (Stx2)were detected in the 10 tested strains by PCR. They also said that the Shiga toxin-producing *E. coli* (STEC) represented a great risk to public health, and also concluded that the *E. coli* strains harbored Shiga toxin (Stx1 and Stx2) and that was agreed with our study. Also***, Hamza et al. (2016)*** detected (Stx1) in all *E. coli* strains isolated from broiler chickens (100%) and (Stx2) in 17 strains (47.2%) which disagreed also with our study.

CONCLUSION

The broiler chickens infected by Non – O157 Shiga toxin producing *E. coli* (STEC), may consider a severe hazard. The PCR assay of (Stx1) and (Stx2) genes is a more practical and reliable method for molecular epidemiological studies of STEC strains because of its more accurate ability to determine. Broiler chickens

should be considered as a reservoir of STEC and should be careful during dealing with broiler chickens which suspected infected by the STEC.

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