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Shiga toxigenic Escherichia coli isolated from table egg

Marwa, M.N. EL-Gendi and Manal, M. Amin

Animal Health Research Institute (Assiut Provincial Lab.) Food Hygiene

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) represent a severe public health issue worldwide, causing life-threatening diseases in the human gastrointestinal tract. All collected samples (40 samples for each balady and poultry farm eggs) were initially subjected to bacteriological examination for enumeration and isolation of *E. coli*, serotyping, and then subsequently, the isolates were exposed to polymerase chain reaction application. *E. coli* could be enumerated in 6 samples of balady egg shells in percentage of 15% (6 samples) with an average 5.6 cfu / ml. Overall, 18.75% (n= 15 /80) samples were found to be contaminated with one or more *E. coli* isolates. All possible *E. coli* colonies (n=15) appeared on Eosin methylene blue agar plates during the screening process were picked for further analysis. Among the suspected isolates, 6 were confirmed as *E. coli*, which were further serotyped using polyvalent *E. coli* antisera. In this study, 6 different *E. coli* serotypes namely O111:H5, O111:H21, O119:H2, O27:H7, O153:H5 and O1:H21 were identified. Out of these 6 serological strains, 1 strain (O119:H2) was positive for *stx2* gene and isolated from balady egg shell. The eggs contaminated with Shiga toxin-producing *E. coli* should therefore be taken with caution and the public should be educated on the dangers associated with consumption of raw and under cooked eggs and egg products, retailers should be encourage to store their eggs in refrigerators and practice good hygiene in order to prevent microbial growth on the eggs. Also, hygienic measures should applied in home produced balady eggs to lower bacterial load in egg shell and subsequently in egg content.

Keywords: *Escherichia coli, Shiga toxin, table egg.*

Introduction

Escherichia coli (*E. coli*) is normally found as nor-mal flora in the intestinal tract of human and warm-blooded animals, but some strains have acquired pathogenic or toxigenic virulence factors that make them virulent for human and animals (**Malik and Memona 2010)**. *E. coli* is Gram-negative, facultative anaerobe, rod-shaped bacterium and the normal habitat in the lower intestine of warm-blooded organisms (**Singleton, 1999**). Most *E. coli* strains are harmless, but some serotypes are pathogenic and causes serious diseases in human and the main source of contamination is fecal oral route and can cause food poisoning as severe abdominal cramps, diarrhea in addition to urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains have a major role in bowel necrosis (**Todar, 2007**). *E. coli* is harmful bacteria that consists of several strains as enteropathogenic (EPEC), enterohaemorrahgic (EHEC), enteroinvasive (EIEC), Shiga toxin-secreting (STEC), enterotoxigenic (ETEC), diarrhea associated hemolytic (DHEC), enter aggregative (EAAggEC) and cytolethal distending toxin recreating (CDTEC) that have side effects on the health. Infections with *stx* producing *E. coli* leads to hemorrhagic colitis in addition to hemolytic uremic syndrome (**Butler, 2012**) that leads to thrombocytopenia

and renal injuries (**Noris and Remuzzi, 2005**). The initial symptoms of STEC infection are abdominal pain, cramps and non-bloody diarrhea, which observed after incubation period 3-5 days (**Griffin, 1995**).

STEC strains produce two powerful phage-encoded cytotoxins causing tissue damage in humans and animals, called Shiga toxins or verotoxins (*stx1*/VT1 and *stx2*/VT2), which are the common feature and main virulence factors of STEC and are directly correlated with human pathogenicity (**Lindgren *et al.,* 1993**). *Stx2* is the most powerful toxin, and the toxin producing strains are usually associated with more severe infections (**Muniesa *et al.,* 2004 and Gyles, 2007**). In addition, some STEC strains can tightly attach and form attaching and effacing lesions to intestinal epithelial cells through an adhesin called intimin.

The egg shell becomes contaminated during contact with environmental sources as dust and faeces (**Board and Tranter, 1995).** The eggs can be acquired infection or contamination through shell (horizontally) or vertically (transovarially). Presence of coliform and enterobacteriaceae give better indication and analysis about the hygienic quality of eggs (**Roberts *et al.,* 1995**) and suspected public health hazard from eaten raw eggs.

This study was adopted on enumeration and isolation of *E. coli* and detection some virulent genes of *E. coli* as *stx* (*stx1, stx2*).

Materials and Methods

A) Collection of samples: A total of 400 random eggs, representing 80 samples, (40 from balady egg, and 40 from poultry farms) were collected from, poultry farms, groceries, framers, supermarkets and rural area located in Assiut Governorate, Egypt. Each egg sample (composed of 5 eggs) was placed in a sterile plastic bag and dispatched to the laboratory with a minimum of delay where they were prepared and examined.

B) Preparation of samples (APHA, 1992):

1. Egg shells: Egg shell was washed by a surface rinse method as described **by Moats (1980) and APHA (1992)** where each egg sample was immersed In 100 ml of 0.1 sterile peptone water in a jar and shaken for 15 min on a mechanical rotary shaker. The obtained rinse solution from the five eggs of each group was combined.

2. Egg content: The egg was prepared for evacuation of its content according to **Speck (1976) and APHA (1992).** Each egg was washed with warm water (32ºC) using a brush and soap, the egg was drained and immersed in 70% Alcohol for 10 min, then flamed after it has been removed from alcohol. A hole was made in the blunt end of the egg by using sterile scalpel. The contents of each group (sample) were removed aseptically and received into a sterile mixer until the sample becomes homogenous.

C) Preparation of serial dilutions (APHA, 1992): Ten-fold serial dilutions up to 10^3 were aseptically prepared from the rinse solutions, as well as from the homogenous egg contents using 0.1% sterile peptone water.

D) Microbiological examination:

1) *E- coli* count (MPN/ml): (FAO, 1992): Total fecal coliforms and *E. coli* using three tubes Most Probable Number (MPN) technique was employed. MPN was streaked on to Eosin Methylene Blue (EMB, OXOID, England). Typical isolates of *E. coli* were confirmed based on their IMVIC pattern according to **Koneman *et al.* (2005).**

2) Isolation of *E. coli* (ICMSF, 1998): Pre-enrichment: from the original dilution, one ml was inoculated into MacConkey broth tubes supplemented with inverted Durham's tubes. Inoculated tubes were incubated at 37°C for 24 hours. **Enrichment broth:** One ml from positive MacConkey tube was inoculated into another MacConkey broth tubes and incubated at 44°C for 24 hours. **Plating media:** Loopfuls from positive MacConkey broth tubes were

separately streaked onto Eosin Methylene Blue agar medium (E.M.B.), which was then incubated at 37°C for24 hours. Suspected colonies were metallic green in color. Suspected colonies were purified and inoculated into slope nutrient agar tubes for further identification.

Identification and characterization of isolated strains:

All isolates were refreshed onto nutrient ager and incubated at 37°C for 24 h. Suspected colonies of *E. coli* were exposed to biochemical testes as Oxidase, Catalase, Indole, Methyl red, Vogus proskouer. Citrate utilization, Hydroben sulphide test and urease test according to **Kreig and Holt (1984).**

Serological identification of *E. coli*:

This part has been done in Serology Department in Animal Health Research Institute, El-Giza, Egypt. Positive biochemical isolates were subjected to serological identification. The isolates were serologically identified according to **Kok *et al.* (1996)** by using ployvalent *E. coli* antisera.

Molecular identification of STEC isolates:

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 56°C 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 Primer:- Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification.

For *stx1*, *stx2* duplex PCR, primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix **(Takara, Japan)**, 1 µl of each primer of 20 pmol concentration, 13 µl of water, and 8 µl of DNA template. The reaction was performed in an Appliedbiosystem 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, 40 µl of each duplex PCR product were loaded in each gel slot. Generuler 100 bp DNA ladder (Fermentas, Thermo) were 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 (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Stx1	ACACTG-GATGATCTCAG TGG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Dipineto *et al.*, (2006)**
	CTGAATCCCCC TCCATTATG							
Stx2	CCATGACAAC-GGACAGCAGTT	779						
	CCTGTCAACTG AGCAGCACTTT G							

Results

Table (2). Statistical analytical results of the examined samples of eggs shells and contents based on their fecal coliform counts (MPN /ml**).**

Egg samples	Number of samples	Positive samples		Counts / ml		
		No.	%	Minimum	Maximum	Average
Balady egg shells	40	12	30%	<3	$2.1X10^2$	1.7X10
Poultry farm egg shells	40	10	25%	<3	$1.5X10^2$	1.1X10
Balady egg contents	40	7	17.5%	<3	$2.4X10^2$	6.9
Poultry farm egg contents	40	5	12.5%	<3	4.3X10	2.6

Table (3). Statistical analytical results of the examined samples of eggs shells and contents based on their E. coli counts (MPN /ml).

Egg samples	Number of samples	Positive samples		Counts / ml		
		No.	%	Minimum	Maximum	Average
Balady egg shells	40	6	15%	<3	9.1x10	5.6
Poultry farm egg shells	40	0	0%	<3	<3	<3
Balady egg contents	40	0	0%	<3	<3	<3
Poultry farm egg contents	40	0	0%	<3	<3	<3

Table (4). Prevalence of *E. coli* isolated from examined table eggs:

Egg samples	Number of samples	*E. coli* isolates	
		No.	%
Balady egg shells	40	6	15%
Poultry farm egg shells	40	0	0
Balady egg contents	40	0	0
Poultry farm egg contents	40	0	0

Table (5). Serotypes of isolated *E. coli* strains from examined balady egg shells:

Serotypes	No of strains	No. of E. coli contaminated samples			
		Balady egg		Poultry farm egg	
		Shells	Contents	Shells	Contents
O111:H5	1	1	0	0	0
O111:H21	1	1	0	0	0
O119:H2	1	1	0	0	0
O27:H7	1	1	0	0	0
O153:H5	1	1	0	0	0
O1:H21	1	1	0	0	0

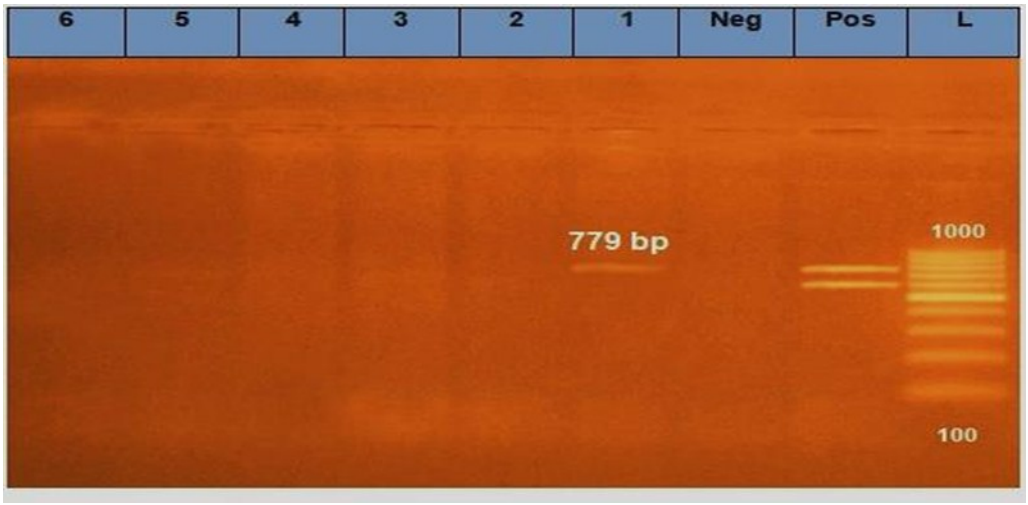

Figure (1). Agarose gel-electrophoresis *stx1* and *stx2* genes of Escherichia coli. Duplex PCR of *stx1* (614-bp) and *stx2* (779-bp) genes of *E. coli*. Lane L: ladder, Lane Neg: negative control, Lane Pos: positive control, Lanes 1: positive strains for *stx2* gene.

Discussion

Food-borne diseases caused by micro-organisms are a large and growing public health problem (**Casey *et al.*, 2012**). Contamination of eggs and egg products with microorganisms can affect egg quality, which may lead to spoilage and pathogen transmission. This might induce food borne infection or intoxication to consumers. Today, eggs remain a staple food within the human diet, consumed by people throughout the world. They are consumed worldwide in the form of pastries, stews and beverages and are considered very nutritious and a cheap source of protein (**MAFF, 2000**).

According to the results reported in ***Table 2*** it was found that fecal coliforms were present in 30%, 25%, 17.5% and 12.5% of balady egg shell, poultry farm egg shells, balady egg contents and poultry farm egg contents samples, respectively with an average counts of 1.7×10, 1.1×10, 6.9 and 2.6 cfu/ ml, respectively. Our results are in accordance with that obtained with **EL-Kholy *el al.*, (2014).** These results were lower than those obtained by **Anand *et al.* (1994)**, while are higher than those of **Refaat (2009)** for egg shell. From the obtained results it is apparent that the counts of fecal coliforms isolated from egg shells were higher than those from egg contents because the shells are more liable to be contaminated. This agrees with **USDA (2011)**, who mentioned that micro-organisms can be found on the outside and inside of the egg shell. This may be due to the fact that the egg emerges from the hen's body through the same passageway the faeces is excreted, micro-organisms inside an un-cracked

egg or intact egg may be due to the presence of pathogen within the hen's ovary or through oviduct, before the shell forms around the yolk and albumin. Faecal contaminants could also occur through the pores on the shell after they are laid. **Ansah *et al.* (2009)**, reported that, as eggs stay longer, their resistance reduce enabling these organisms to penetrate into the egg content. Warm and moist litters, poor condition in the farm houses were reported to be sources of fungi growth and sporulation (**Abdullahi, 2010**). Several factors have been implicated in egg contamination. Among these are faeces of the birds, litter material, improper handling of the eggs by retailers, unhygienic conditions of the markets where these eggs are being sold, contaminated egg crates, packing and poor storage method (**Bruce and Drysdale, 1994**). Others are cloths and hands of poultry workers, the environment, dust transporting marketing and weather condition. Eggs in many stores were exposed to high temperature and low humidity which favors the growth of microbes and result into rapid decrease in the quality of eggs.

The summarized results in **Table 3** revealed that *E. coli* were enumerated only in 15% of balady egg shells with mean values of 5.6 cfu/ ml. Also, **EL-Kholy *el al.,* (2014)** could detect *E.coli* in 14.71% of poultry farm egg shell and 11.76% of contents with mean counts of 1.1×10 and 0.85cfu/ ml. In our search *E. coli* failed to be enumerated and isolated from the rest of the examined samples. These results were lower than those recorded by **Petrak *et al.* (2000**) for egg content and **Akhtar *et al.* (1982)** for egg shell and content, while, the obtained results were higher than those of **Refaat (2009)** for egg shell and **EL-Leboudy and EL -Mossalami (2006)** for egg shell and content. *E. coli* is the major micro-organism isolated from the surface balady egg shells in the study, this might be attributed to the fact that *E. coli* are normal inhabitants of intestinal tracts of birds (**Singleton and Sainsburg, 1981**). They have also been known to contaminate the surface of egg while the mechanical process can spread the bacteria through the eggs. Contaminations with the pathogen while in the field occur through improperly decomposed manure and poor hygienic practice of farm workers. *E. coli* can bring about urinary tracts infections, pneumonia meningitis and peritonitis in humans (**Schoeni and Doyle, 1994**).

From the obtained results it is apparent that the counts of fecal coliforms and *E. coli* isolated from egg shells were higher than those from egg contents because the shells are more liable to be contaminated. *E. coli* is one of the major problems in chicken production influencing heavier losses and sever drop in egg production, about 5.5 % mortality and 10-20% drop in eggs was observed with *E. coli* infections **Qu *et al.* (1997).**

The summarized results in **Table 4** revealed that *E. coli* could be isolated from 6 samples of balady egg shells in a percentage of 15% and failed to be isolated from all other samples. The results of this study do not harmony with the prevalence of *E. coli* in Australia eggs where 60.78% of eggs were contaminated with *E. coli* (**Gole *et al.,* 2013**). **Eman and Saad, (2015)** isolated *E. coli* in a percentage of 28.58% from examined leaking chicken table eggs. **Sabrinath *et al.* (2009)** documented that 13.3%, 45.8% of egg contents collected from large farms and small farms in Grenada were contaminated with *E. coli*, respectively. **Cortés *et al.* (2004)** presented that 45% of eggs were contaminated with *E. coli*. **Stępień-Pyśniak (2010)** showed that 4.3% of egg shells were contaminated with *E. coli* while 19% of egg contents in Shahrekord, Iran were contaminated with *E. coli* (**Safaei *et al.,* 2011**). On the other hands, these results are in agreement with the report of **Al-khalaf *et al.* (2009)** who reported that 10% of egg shells were contaminated with *E. coli* while 12% of unwashed Baladi egg shells were contaminated with *E. coli* (**Bahobail *et al.,* 2012**). Almost similar results were reported by (**Arathy *et al.,* 2011**) they could detect an overall isolation rate of 12.2%, while 8% of the isolates were detected from shell and 5% from yolk samples, respectively.

Higher isolation rate was reported by **Adesiyun *et al.,* (2005)** who recorded 37.0% as an overall isolation rate, 28.3% as an isolation rate from egg shell, while they recorded almost similar isolation rate from egg content samples (3.8%). Lower isolation rates were recorded by **Saitanu *et al.,* (1994)** who isolated *E. coli* from egg shells and in egg contents with a rate of 3.5% and 1.2%, respectively. **Samah *et al.,* (2015)** declared a total of 36 *E. coli* isolates with an overall prevalence rates of 18%. Among the total isolates, 21 (10.5%) isolates, 9 (4.5 %) isolates, and 6 (3.0%) isolates were detected from the shell, egg contents, and both shell and contents, respectively.

Serotypes of *E. coli* are classified according to the Kauffmann scheme, currently there are approximately 180 O, 60 H and 80 K antigens; The numbers change as new ones are identified and previous ones that are duplicated or attributable to another bacterial species are removed, additional serotypes with O antigens that have not been recognized also are found in most surveys. Even though molecular methods for identifying specific virulence genes are available, serotyping remains a useful tool for epidemiologic studies. Serotyping provides a means of relating previous work with new work. Variations in the distribution of serotypes according to geographic region occur. Many other serotypes have been found less frequently, while some APEC do not belong to known serotypes or are untypeable (**Swayne, 2013**).

In the present study (**Table 5**), 6 serotypes belonged to 5 different O groups were identified, of which O111 predominated with an isolation rate of (33.33%), while, O119, O27, O153 and O1were isolated with a rate of (16.66%), each. Like other studies performed in Egypt (**Maha 2013, Samah *et al.,* 2015 and Elafify *et al.,* 2016**), the non-O157 serotypes (O125, O26, O111, O145, and O103) were most commonly present in food products. Where, **Maha (2013)** isolated *E. coli* strains from different types of table eggs and were serotyped into 7 different serotypes included O44, O111, O114, O125, O126, O127 and O128. Most of these isolates (37/39) were *stx2* positive, while, **Elafify *et al.,* (2016)** found that 18% (n=18/100) of table egg samples were contaminated with one or more *E. coli* isolates and 9 different E. coli serotypes namely O78, O114, O2, O44, O1, O125, O128, O124 and O26 were identified. **Elafify *et al.,* (2016)** documented that out of 9 serological strains, 3 strains (O44, O1 and O128) were positive for *stx2* gene. The prevalence of such contaminants may be attributed to the poor hygiene in the resulting areas; consequently such eggs with high coliforms constitute an economic and public health importance (**Sabreen, 2001**).

Our search results revealed that the most contamination of *E. coli* occurred in the shells as a several factors were implicated in egg contamination due to the principle way of contamination occurs in short time after laying as the egg shells become within contact environment as soil, dust and dirty nesting material (**Smith *et al.,* 2000; Ellen *et al.,* 2000**). Also, the growth temperature of this organism is 37^{o}C and mostly isolated from the environment or from the intestinal tract of vertebrate animal and most of bacterial contamination of eggs contents result from bacterial contamination of egg shells that invade egg contents under improper conditions. Beside the horizontal way egg may contaminated vertically or through ovary and oviduct (transovarially) (**Bruce and Drysadale, 1994**). The presence of *E. coli* in the eggs indicates poor or improper hygiene (**Kornacki and Johnson, 2001; Ricke *et al.,* 2001; Nazir *et al.,* 2005a)**.

None of the 6 tested *E. coli* isolates showed positive PCR results for the *stx1* gene (Fig. 1). However, 1 from 6 isolate of *E. coli* (16.7 %) was recorded as positive for the *stx2* gene. A close result was reported by **(Samah *et al.,* 2015** and **Zahraei *et al.,* 2007)** whose suggested that *stx2* may be widespread among APEC as they detected *stx1* in 0% and one isolate (8.33%) out of the 13 and12 tested isolates, but *stx2* was detected in 5 isolates (38.46%) and 9 (75%) isolates. **AL-Ashmawy,**

(2013) has much correlated results as she detected *stx2* in 37/39 of the *E. coli* isolates from table eggs. The correlation was high also because none of the positive *stx2* isolates showed positive results for *stx1* gene. The heat stable toxin (*stx*) causes disruption of chloride channels in the cell and secretion of fluid and electrolytes into the intestinal lumen causing diarrhea (**Gaastra and Svennerholm, 1996**). Egg shell quality is of primary importance to the egg industry worldwide. Egg shells need to be strong enough to remain intact throughout the chain from the time that the egg is laid until it is used by the consumer (**Roberts, 2010**).

Conclusion

Due to the worldwide consumer demand for eggs, periodic assessment is required to offer safe and good quality eggs for consumption, where the environmental condition combined with poor hygiene that characterizes poultry or egg production favors survival and proliferation of micro-organisms. Our findings underscore the need for optimum hygienic practices in farms in order to decrease bacterial load in balady chicken eggs. Marketers should also ensure good hygienic standard at various retail outlets as indicated with the isolation of *E. coli* specie. Hence consumption of raw and under cooked eggs or egg products should be discouraged. In conclusion, our study confirm that balady eggs which are consumed as food can harbor bacterial pathogen of zoonotic importance, *E. coli* pathogens may impose public health hazard. The study recommends rising public awareness to the importance of proper thermal processing and cooking of eggs specially for immune-compromised group as pregnant women, children and old ages. The study also recommends regular monitoring and surveillance of house hold sector together with guidance programs to the public targeting rising awareness for safe house hold rearing procedures of chicken, storage and handling of eggs, in order to prevent dissemination of dangerous pathogens through environment and the transfer of infection to other animals and human.

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