ADVANCES IN ENVIRONMENTAL BIOLOGY

2022 January; 16(1): Pages 1-12
https://doi.org/10.22587/aeb.2022.16.1.1

ORIGINAL ARTICLE AENSI PUBLICATIONS

Detection of SHIGA-TOXIN producing E. coli in some retail markets in Egypt using qPCR assay with special reference to serotyping

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Received date: 14 December 2021, Accepted date: 22 January 2022

Cite as: M.A. Abdelmonem., M. A. Kelany., M. Fawzy., R. sheta., A. Ageez., A. A. Ismail., S. I. A. El-Moez., 2022. Detection of SHIGA-TOXIN producing E. coli in some retail markets in Egypt using Qpcr assay with special reference to serotyping. Advances in Environmental Biology, 16(1): 1-12. DOI:10.22587/aeb.2022.16.1.1.

Abstract

Background: Shiga toxin-producing Escherichia coli are dangerous foodborne pathogens that represent a severe public health issue worldwide. Raw foods are considered an important source of STEC infection in humans. Objective: In the current study, STEC contamination was investigated in 80 raw foods (chicken, beef, and milk) and water collected from different localities of Greater Cairo using Real-Time PCR. Moreover, the virulence genes of isolates were characterized. Results: STEC was detected in eight samples, (7 beef and 1 chicken), which represent about 10% of the tested samples. Positive beef and chicken samples show the presence of 13 and 2 STEC genes, respectively. Five samples were positive for eaeA (intemin), two samples were positive for stx1 gene and eight samples were positive for stx2 gene. Beef samples reveal the highest incidence of virulence gene stx2 (35%), followed by eaeA (20%), then stx1 (10%). The incidence of STEC was lower in chicken samples and the prevalence of virulence gene was 5% for stx2 and eaeA, respectively. Stx2 gene was the most prevalent subtype identified in beef samples. Serotyping of isolated STEC strains (14) revealed isolation of seven STEC strains belong to O157 serogroup, two strains belong to O111and five strains belong to serogroup O26. Conclusion: The current study concluded that recovery of STEC from raw chicken and beef samples is of important concern.

Keywords: Shiga toxin-producing E.coli (STEC), Foodborne pathogens, RT-PCR, eaeA, stx2, Serogroup

INTRODUCTION

Safe food is essential for human survival. However, some foods can pose risks to human health and threaten life. Science plays a critical role in making food safety decisions through risk assessment through the determination, evaluation, analysis and identification of hazards. The dangers include microbiological pathogens, environmental pollutants, excessive consumption of dietary supplements and toxins [1]. Food is a good habitat for bacteria to grow, which leads to food poisoning. Contaminated food is life-threatening all over the world. Accordingly, the government follows the instructions of the World Health Organization (WHO) to prevent infections from food contamination [2]. WHO stated five solutions for safer food, including, processed and raw food should be distant in storage, food must be maintained at an appropriate temperature, hands should be washed before handling

food to keep clean, cooking should be carefully done using suitable temperature and the use of safe raw materials and water without infection [2]. Microorganisms can survive a wide range of environmental conditions [3]. Food microbiology includes studying microorganisms' beneficial and harmful effects on eggs, poultry, and processed/raw meat [4]. Food microbial contaminant causes food spoilage and poisoning, resulting in severe public health problems and causing deaths and illnesses. Therefore, microbiological analysis is vital to determine the safety and quality of food.

Food poisoning is a disease caused by the ingestion of contaminated foods. Some populations are more susceptible to illness and can't overcome pathogenic infection through natural barriers [5]. According to the Centers for Disease Control and Prevention (2004) [6], bacterial foodborne pathogens include *B. cereus, B. anthracis, C. jejuni, E. coli O157:H7, Salmonella spp. and S. aureus.* Food production leads to the contamination of food with many microorganisms that can negatively affect human health [7]. As a result, many people die each year due to illnesses associated with food poisoning [8]**.**

Escherichia coli (E.coli) are among the most studied bacteria for food poisoning. *E.coli* strains can be divided into six groups/pathotypes, probably derived from the transmission of coding plasmids containing pathogenicity genes from other species, creating strains with the capacity to initiate serious diseases. *E.coli* groups include Enteropathogenic *E.coli* (EPEC), Enteroaggregative *E.coli* (EAggEC), Enterotoxigenic *E. coli* (ETEC), Attaching and effacing *E.coli* (A/EEC), Enteroinvasive *E. coli* (EIEC) and Enterohemorrhagic *E. coli* (EHEC) [9].

Pathogenicity STEC is due to the virulence of Shiga-like toxins belonging in their cytotoxicity, which allows them to induce cell death by blocking the ability of cells to synthesize proteins. This mechanism ends with cell death (apoptosis) [10]. Shiga-toxin-producing *E. coli* O157:H7 outbreak was first reported in January 1993 and was associated with the consumption of undercooked hamburgers. In total, 501 cases were reported, 477 were culture-confirmed. *E. coli* O157:H7 was confirmed to be stx+, eaeA+, and exhA+ [9]**.**In 1999, thousands of New York habitats were infected with STEC O157: H7 after drinking water contaminated with cattle manure. In the last decade, the Centers for Disease Control and Prevention (CDC) have reported STEC outbreaks traced back to sprouts, hazelnuts, lettuce, poultry, beef, frozen pizza containing contaminated pepperoni, ready to eat salads, cookie dough, cheese and organic spinach [11]**.**

Recently PCR has become an essential technique for detecting bacteria, as the DNA from a single living or dead bacterial cell can be amplified in 1hr, which is relatively rapid and accurate compared with other methods [12]**.** Therefore, Multiplex PCR and real-time PCR are the most popular. Real-time PCR allows reactions to be characterized by the time when amplification of the PCR product is first detected using primers and a fluorogenic probe specific to the serogroup-associated genes [12]. Moreover, it compromises sequential steps such as microbial enrichment, nucleic acid extraction, detection of virulence genes, detection of serogroup-associated genes, and finally, isolation from positive samples [13].

The current study aims to determine Shiga-toxin-producing E. coli (STEC) in different food and water samples. The second aim is to identify virulence genes from the STEC isolates, genes encoding for the production of Shiga toxins (stx) in their genomes (stx1 andstx2) and eaeA. Another aim of the current study is to identify the serotyping of the isolated strains hindering virulence genes. This study links the genomic diversity responsible for the varying degrees of pathogenicity to the serogroups of STEC isolates from collected samples (milk, water, beef, and chicken). The current study was carried out on 80 samples collected from different vendors all over Greater Cairo, which were investigated and analyzed using real-time PCR, traditional culturing method, and serological testing

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MATERIALS AND METHODS

Equipment Chemicals, reagents and suppliers; are all supplied from an accredited lab by FINAS(QCAP). Sample collection, 80 raw samples were randomly collected from farms, butchers, and retail markets in different localities of Greater Cairo. These samples were categorized as 20 water samples, 20 milk samples, 20 beef samples, and 20 chicken samples.

Culture media; Modified tryptone-soy broth (mTSB) (LabM), Buffered peptone water (Oxoid), TBX Agar (Oxoid), STEC CHROM™ agar (Oxoid), Tryptone Soya agar (Oxoid), Cefixime tellurite Sorbitol MacConkey agar (CT-SMAC) (Lab M), all media are prepared according to manufacture instructions autoclaved at 121ºC.

Test portion and initial suspension; samples were prepared in enrichment medium to give 10-1 of the original test portions final dilution. According to matrix samples, 25 g of solid or liquid sample (beef, chicken, milk, or water) were aseptically transferred to a sterile conical flask containing 9x ml of mTSB to which novobiocin was added. On the other hand, according to matrix samples assumed to contain stressed target bacteria, the frozen products (beef and chicken) were left to thaw at room temperature, and then the test portion (25 g or 25 ml) was transferred to a conical flask containing 9x ml of BPW.

Enrichment and nucleic acid extraction; the conical flasks were incubated at 37˚C ± 1℃ for 18 to 24 hours. Following incubation, an appropriate nucleic acid extraction kit (PrepMan™ Ultra sample preparation reagent kit) for Gram-negative bacteria was used for all the 80 samples. After incubation, 1 ml of the enrichment culture was taken using a sterile pipette, added in an

Eppendorf and placed in the centrifuge for 3 mins at 15000 rpm. Following centrifugation, the supernatant was removed and 100 µl of lysis buffer (Prepman™ Ultra- Applied Biosystems) were added to the Eppendorf. After that, the Eppendorf was placed in the thermal blocker for 10 mins at 95˚C, and placed again in the centrifuge for 3 mins at 15000 rpm. Finally, the supernatant was used in order to perform the dilutions.

Dilution of sample; two dilutions were made for each sample10^{-2}. Two Eppendorf tubes were used with 90 µl of nuclease-free water was placed in each one. 10 µl of the sample was placed in the first Eppendorf, homogenized 10^{-1}, and then 10 µl were transferred from the first Eppendorf and placed in the second one to obtain dilution 10^{-2}.

PCR amplification is a standard sequence; the described PCR amplification approach is based on real-time PCR (PikoReal 96). Specific primers and probes were used and considered as reference reagents, and they are listed in the following table (**Table 1**).

Table 1: Degenerate primers and probes used for 5'-nuclease PCR assays to identify *E. coli*.

Target gene	Forward primer, reverse primer and probe sequence (5'-3') a	Amplicon size bp	Location within sequence	GenBank accession No.	Reference
stx1	• Forward: TTT GTY ACT GTS ACA GCW GAA GCY TTA CG • Reverse: CCC CAG TTC ARW GTR AGR TCM ACR TC • Probe-FAMCTG GAT GAT CTC AGT GGG CGT TCT TAT GTAATAMRA	131	878 to 906 983 to 1008 941 to 971	M16625	[51]
stx2b	• Forward: TTT GTY ACT GTS ACA GCW GAA GCY TTA CG • Reverse: CCC GAC TTC ARW GTR AGR TCM ACR TC • Probe-HEXTCG TCA GGC ACT GTC TGA AAC TGC TCCTAMRA	128	785 to 813 887 to 912 838 to 864	X07865	[51]
eaeA	• Forward: CAT TGA TCA GGA TTT TTC TGG TGA TA • Reverse: CTC ATG CGG AAA TAG CCG TTA • Probe-CY5ATA GTC TCG CCA GTA TTC GCC ACC AAT AACTAMRA	102	899 to 924 1000 to 979 966 to 936	Z11541	[52]

a In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).
b This combination of primer/probe recognizes all the stx2 variants except stx2f.

The STEC Real-time PCR reaction mixture; real-time PCR amplification was performed using (ProMag STEC Screening QPCR Diagnostic Kit - Berlin). The kit comprises STEC QPCR premix, STEC positive control, and STEC negative control (PCR grade water). 15 µl of STEC mix was placed in the real-time PCR tube and 5 µl of the DNA sample was added to the tube (total = 20 µl per tube).

Detection and interpretation of PCR products; the real-time PCR assay used allowed the detection of the STEC genes using different primers as shown in **table 1**. The PCR results obtained are interpreted by the software linked to the apparatus (PikoReal software 2.2). During amplification, the software monitors 5' nuclease PCR amplification by analyzing fluorescence emissions of the reporter dye for each sample. This method was mainly responsible for detecting and scanning 3 main genes: FAM (target for *stx1* gene), HEX (target for *stx2* gene), and Cy5 (target for *eaeA* gene). The thermal conditions of the PCR were as follows: 40˚C 5 min, 95˚C 2 min "One cycle" 95˚C 10 sec, (60˚C 30 sec) * 40 cycles.

Isolation of STEC strains (positive enrichment); identifying the STEC strains is required to confirm that the positive PCR signals are generated from genes presented in the same live bacterial cell. The procedure of the isolation was performed as follows: In the case of the positivity to one of these genes (*stx1, stx2, eaeA)*, the enrichment cultures of the positive samples were returned back to, point-inoculated on Tryptone soy agar or TBX (non-selective media), STEC CHROM agar and O157 CHROM agar (selective media), and PrepManUltra™ (colonies may be pooled in water to a total of 10 per pool) and placed in the incubator for 18-24 hrs at 37°C. After that, Stx-coding genes were detected on the isolated colonies or the PrepMan Ultra pools (using real-time PCR). When the pool results turned out to be positive, the Tryptone soy agar was returned to individual assay colonies that formed the positive pool and hence, one positive colony was selected. The colonies were then identified as STEC. However, when the pool results turned out to be negative, this was identified as presumptive detection of STEC.

Sero-grouping identification; the serogroup detection was performed for the obtained isolates of the samples positive for *stx* and the *eaeA* genes using real-time PCR (7500 real-time PCR system). The *E. coli* isolates were serogrouped based on their 'O' antigen with specific primers, as shown in **Table 2**.

Table 2: Primers and probes used to amplify O antigen-specific genes in 5'-nuclease PCR assays.

Target gene	Forward primer, reverse primer and probe sequence (5'-3')	Amplicon size Bp	Location within sequence	GenBank accession No.
***rfbE* (O157)[51]**	• Forward: TTT CAC ACT ACT TAT TGG ATG GTC TCAA • Reverse: CGA TGA GTT TAT CTG CAA GGT GAT • Probe-**FAM**AGG ACC GCA GAG GAA AGA GAG GAA TTA ACG**TAMRA**	88	348 to 372 412 to 435 381 to 410	AF163329
***wbdl* (O111)[51]**	• Forward: CGA GGC AAC ACA TTA TAT AGT GCT TT • Reverse: TTT TTG AAT AGT TAT GAA CAT CTT GTT TAGC • Probe-**FAM**TTG AAT CTC CCA GAT GAT CAA CAT CGT GAA**TAMRA**	146	3464 to 3489 3579 to 3609 3519 to 3548	AF078736
***wzx* (O26) [51]**	• Forward: GAC GGC AGA GAA AATT • Reverse: AGC AGG CTT TTA TAT TCT CCA ACT TT • Probe-**FAM**CCC CGT TAA ATC AAT ACT ATT TCA CGA GGT TGA**TAMRA**	135	5648 to 5666 5757 to 5782 5692 to 5724	AF529080
***ihp1* (O145)[51]**	• Forward: CGA TAA TAT TTA CCC CAC CAG TAC AG • Reverse: GCC GCC GCA ATG CTT • Probe-**FAM**CCG CCA TTC AGA ATG CAC ACA ATA TCG**TAMRA**	132	1383 to 1408 1500 to 1514 1472 to 1498	AF531429
***wzx* (0103)[53]**	• Forward: CAA GGT GAT TAC GAA AAT GCA TGT • Reverse: GAA AAA AGC ACC CCC GTA CTT AT • Probe-**FAM**CAT AGC CTG TTG TTT TAT**TAMRA**	99	4299 to 4323 4397 to 4375 4356 to 4373	AY532664

The PCR mix for the serogrouping identification was prepared manually. Forward primer, reverse primer, and probe sequence of each target gene were obtained from (LGC - Biosearch Technologies, UK).

Identification of serogrouping using PCR mix; ten µl of probe master mix, 1 µl of forwarding primer, 1 µl of reverse primer and 1 µl of probe sequence were added to it. 10 µl of the positive sample. Five reactions were prepared to identify the genes listed in **Table 2**.

RESULTS AND DISCUSSION

Surveillance and investigations of Shiga-toxin-producing *E. coli* are essential for food safety and public health. This study performed STEC investigation and analysis using a real-time PCR-based method. The study findings indicate that Shiga toxins and intimin were detected in eight samples collected from the Greater Cairo (one chicken and seven beef), as shown in **Figure 1**. A total of eighty (80) samples of different commodities were analyzed for the STEC virulent genes. It is displayed that STEC virulent genes were present in eight samples with an incidence of 10.13%.

The number of samples analyzed, samples containing STEC, the number of genes detected and virulent genes detected in each commodity (milk, water, beef and chicken) was presented in **Table 3.** Results reveal that STEC virulent genes were not detected in all analyzed milk, water and chicken samples. However, STEC virulent genes were detected in seven of the analyzed beef samples and one chicken sample with virulence gens Stx2 and eaeA in the positive chicken and beef samples with total no of detected genes 2 5, respectively **(Figures 2-4).**

Table 3: Identification of detected STEC

Type of tested samples	Number of samples	STEC positive samples	%	No. of genes detected	%
Milk	20	ND	0	ND	0

Water	20	ND	0	ND	0
Chicken	20	1	5	2	10
Beef	20	7	35	13	65
Total number of samples	80	8	10.00	15	18.75

Overall presence and absence of STEC in raw food samples

The percentage of STEC prevalence is displayed in figure 2. It shows that 9% of the total samples contained STEC. However, 91% of the total numbers of samples were not composing STEC.STEC was present in 5% chicken and 35% beef samples (Figures 3, 4).

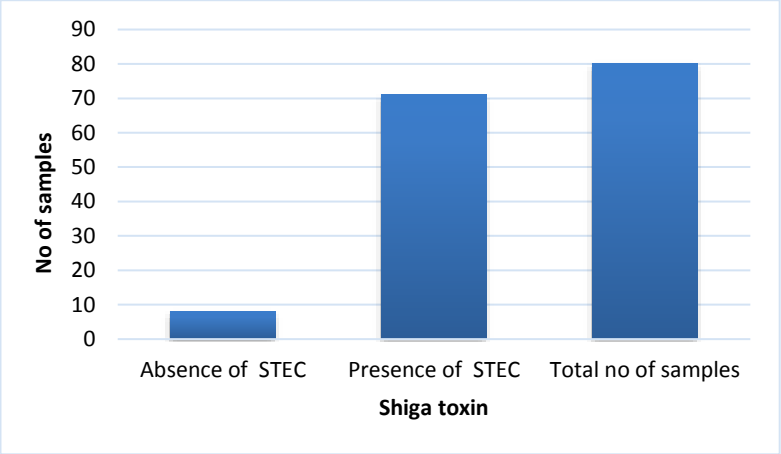

Figure 1: The presence and absence of STEC in food

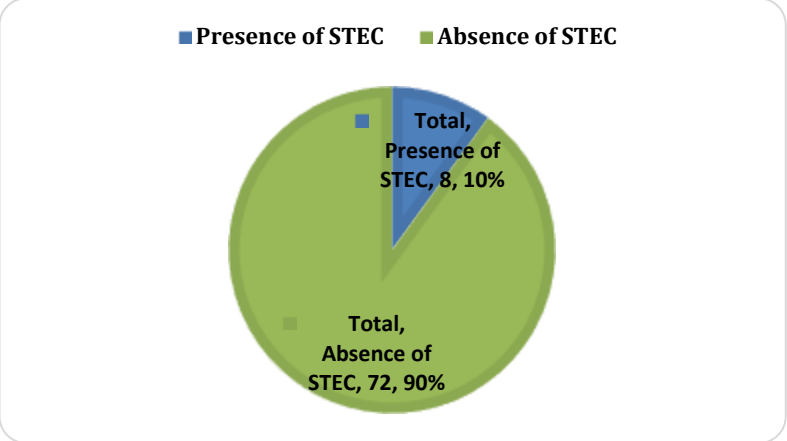

Figure 2: Total no of tested samples (80)

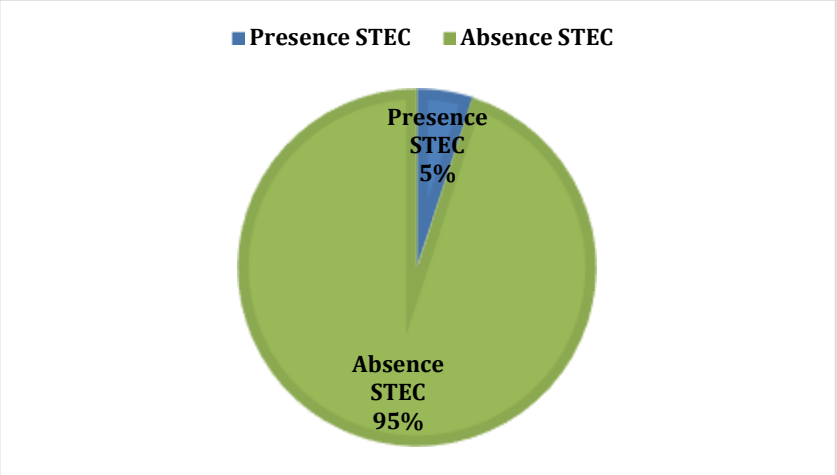

Figure 3: The incidence of STEC chicken samples

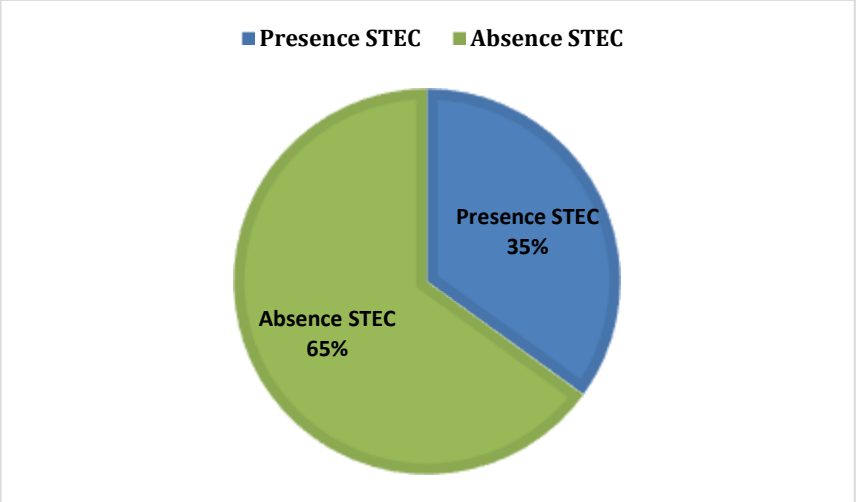

Figure 4: The incidence of STEC beef samples

Prevalence of STEC in tested samples, results of the analyses of 20 milk samples reveal the absence of virulence genes*stx1*, *stx2* and *eaeA* in all tested milk samples. In addition, results reveal the lack of virulence genes *stx1*, *stx2* and *eaeA* in the 20 tested water samples. On the other hand, out of the 20 tested chicken samples, results confirm the virulence genes *stx2* and *eaeA* in one sample and the absence of *stx1*, *stx2* and *eaeA* virulence genes in the other 19 samples. Analysis of the 20 beef samples reveals the occurrence of virulence genes*stx1*, *stx2* and *eaeA* in variable tested samples, the three virulence genes*stx1*, *stx2* and *eaeA* are present in two samples, *stx2* and *eaeA* genes are found in two samples, and*stx2* gene only is found in three samples, as tabulated in **Table 4 and Figure 5.**

Table 4: The occurrence of Shiga toxins and intimin in chicken and beef samples

Virulence genes Commodity (total no 20)	**stx1**	**stx2**	**eaeA**
Chicken 1	-ve	+ve	+ve
Total Chicken	**0**	**1**	**1**
Beef 1	-ve	+ve	+ve
Beef 2	-ve	+ve	+ve
Beef 10	-ve	+ve	-ve
Beef 14	-ve	+ve	-ve
Beef18	+ve	+ve	+ve
Beef19	-ve	+ve	-ve
Beef 20	+ve	+ve	+ve
Total Beef	**2**	**7**	**4**
Total STEC isolates	**2**	**8**	**5**

*-ve = negative, *+ve = positive

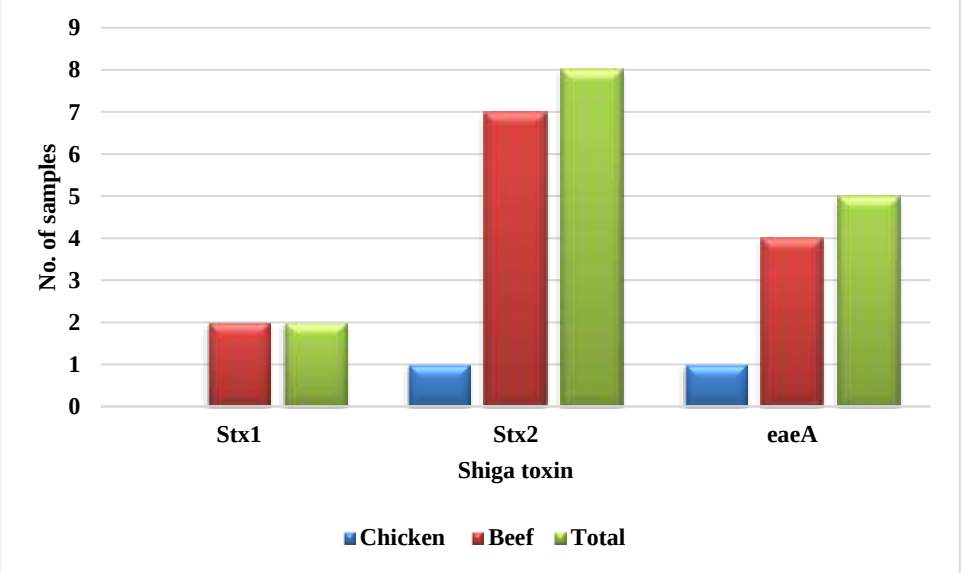

Figure 5: Incidence of Shiga toxins and intimin in chicken and beef samples

Amplification curve of real-time PCR using the real-time PCR product detection apparatus (PIKOREAL™ 96) the real-time PCR amplification curve includes the y-axis representing the fluorescence unit (RFU) and t x-axis representing the number of cycles. The given graphs show the fluorescence recorded in the FAM, HEX and Cy5 PIKOREAL™ 96 detection channels.

Isolation of Shiga-toxin producing *E. coli*, Shiga toxin genes were isolated from the tested positive samples by the subsequent cultural technique. Unfortunately, some isolates were not recovered and couldn't be isolated on media. Shiga-toxin producing *E. coli* appears of the following media.

Out of 80 tested samples, all water (20) and milk (20) samples show negative results for the tested virulence genes. Testing virulence genes reveal the presence of 15 virulence genes, of which 10 were shiga toxin and 4 were intmen. Out of the 20 tested chicken samples, only one sample show positive results for Shiga toxin (*stx*1) and intmen (*eae*A) with an incidence of 5% for each virulence gene. On the contrary, results reveal high incidence of virulence genes in the tested beef samples, shiga toxins were found in nine samples, 2 *stx1* (10%), and 7 *stx2* (35%). Intemin (*eaeA*) was found in 4 beef STEC positive samples with an incidence (20%).

Serotype diversity of Shiga-toxin producing *E. coli*

Detection of serotypes was carried out using real-time PCR for the top five serotypes (O157, O111, O26, O103 and O145) as shown in **Table 5 and Figure 6.**

Table 5: Serotype identification of isolated colonies

Sample number	O157	O111	O26	O103	O145
Chicken 1	+ve	+ve	+ve	-ve	-ve
Chicken total	**1**	**1**	**1**	0	0
Beef 1	+ve	-ve	-ve	-ve	-ve
Beef 2[a]	-ve	-ve	+ve	-ve	-ve
Beef 2[b]	-ve	-ve	+ve	-ve	-ve
Beef 10	+ve	-ve	-ve	-ve	-ve
Beef 14	+ve	-ve	-ve	-ve	-ve
Beef 18	+ve	-ve	+ve	-ve	-ve
Beef 19	+ve	-ve	-ve	-ve	-ve
Beef 20	+ve	+ve	+ve	-ve	-ve
Beef total	6	1	4	0	0
Total	7	2	5	0	0

*Beef 2[a]: isolate no. 1 (beef 2), *Beef 2[b]: isolate no.2 (beef 2)

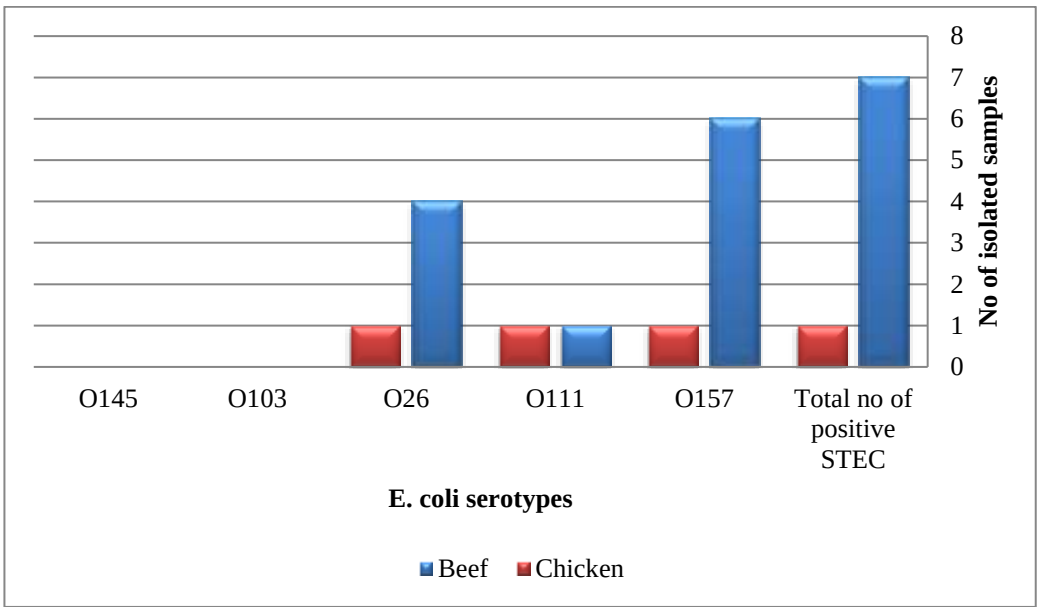

Figure 6: Serotyping of isolated colonies

Real-time PCR amplification curves of the Serotype identification

The real-time PCR amplification was performed using (7500 real-time PCR system) for the serotyping identification of the isolates obtained from chicken isolate 1, beef 1, beef 2^a, beef 2^b and beef 10, respectively. The amplification curves are displayed; blue curve shows the detected target gene (FAM) and the red line shows the passive control (ROX).

DISCUSSION

Shiga toxin-producing *E.coli* or Verotoxin-producing *E.coli* remains a major cause of foodborne-related disease in humans, causing outbreaks of gastrointestinal illness. They have been linked to many disorders in humans, including kidney failure (haemolytic uremic syndrome), chronic arthritis, irritable bowel syndrome and brain and nerve damage [14]**.** Recently, the incidence of foodborne diseases caused by STEC has increased globally. Therefore, STEC outbreaks have become an increasingly important food safety concern. Effectively tackling this problem relies on the availability of sensitive, specific, and reproducible methodologies that can be used for rapid and accurate detection of this pathogen [15]**.** The current study investigates Shiga-toxin producing E. coli in raw samples collected from different localities all over Greater Cairo, especially chicken, beef, milk and water, with seventy-nine samples. This study is performed using real-time polymerase chain reaction (Real-time PCR) as it has minimum sample preparation, high specificity and sensitivity. The qPCR approaches are currently applied in microbiological studies to detect and identify the abundance and expression of a specific target gene which, in this case, the target genes are (*stx1, stx2, and eaeA*). It is a powerful tool for quantifying gene expression levels. Moreover, real-time PCR has higher precision, increased sensitivity (down to one copy), increased dynamic range (greater than 8 logs), and higher resolution (less than two-fold differences) than the traditional PCR [16]**.**

The current study reveals that Shiga-toxin-producing E. coli was detected in some beef and chicken samples as shown in Table 3, Figures 3 &4. STEC was only present in fifteen samples among the eighty investigated samples (beef, chicken, etc. water and milk). Moreover, STEC was absent in all tested water and milk samples and this is shown in **Table 3, Figures 1&2,** respectively. Results show the presence of three virulent genes was detected throughout the whole investigation (*stx1, stx2* and *eaeA*). Chicken STEC isolates includes virulence genes,*stx2* (1) & *eaeA* (1) reveals. Beef STEC isolates include virulence genes,*stx1* (2), *stx2* (7) &*eaeA* (4) as shown in **Table 4 and Figure 5**. In addition, eight isolates were successfully obtained and isolated. Twelve out of the twenty-four isolates belong to the subtype *E. coli* O157 and eight isolates belong to the subtype *E. coli* O26and four isolates belong to subtype *E.coli* O111as shown in **Table 5.**

Raw, unpasteurized milk can carry dangerous bacteria such as *Salmonella, E. coli*, and *Listeria*, responsible for causing numerous foodborne illnesses. While proponents have stated that there are benefits to its consumption, the medical community has warned of the dangers. Raw milk availability and regulation vary from region to region [17]**.** The current study showed that STEC was not detected in any of the investigated milk samples **Table 3.** These results agree with [18], who conducted a study using the

immunocapture technique (immunomagnetic separation–IMS) on 153 raw milk samples; none of them hinder the contrary. The current findings disagree with the findings of another [19] who investigated 46 raw milk samples from Canada using an automated fluorescent PCR method, and results reveal 15 samples STEC positive by applying both the culture and the Amplicon-Sensor PCR methods. Our findings contrast with a study conducted by another study [20] in Iran, which reveals that out of 206 raw milk samples, 36 were contaminated with STEC using PCR targeting *stx1* and *stx2* and then *eaeA*. Furthermore, this study does not agree with [21], who studied 106 raw milk samples in Italy using RT-PCR, STEC contaminated revealing26 samples and some were identified as *stx1, stx2* and *eaeA.*

Raw water is water found in the environment that has not been treated and does not have any of its minerals, ions, particles, bacteria, or parasites removed. Raw water includes rainwater, groundwater, water from infiltration wells, and water from lakes and rivers. Drinking water, especially from untreated water supplies, can be a source of STEC strains potentially pathogenic for humans [22]. The current study results showed that STEC was not detected in any of the investigated water samples, as shown in **Table 3.** This goes in agreement with a study done by [23] in the USA, in which 13 water samples were investigated for the presence of STEC using PCR and none of the analyzed samples were identified as STEC.

On the other hand, the current study goes in contrast with a qPCR assay conducted by [24] in Australia on 18 environmental water samples. STEC was detected in 5 samples, in which two samples were positive for *stx2* virulence genes and three samples were positive for *eaeA* virulence genes. Moreover, this study goes as well in contrast with [25], who conducted a study on 650 water samples using multiplex PCR in Brazil. Only 11 samples were positive for *stx* genes and thus considered STEC. In addition, the current research does not agree with [26], who conducted a study in the USA on nineteen water samples collected from lakes and rivers using iCycler iQ5 RT-PCR detection system and ultrafiltration. Five samples were identified as STEC, positive for *stx1* and *stx2* genes.

Chicken is considered the most common type of poultry worldwide due to its relative ease and low raising cost. However, chicken meat can be easily contaminated with *E. coli,* which is easily transmitted to people through undercooked and raw chicken meat [27]. Results of the current study show detection of STEC in one chicken sample hindering two virulence genes *stx2* and *eaeA* **(Figure 3),** revealing three strains serotypes O157, O111 and O26 as shown in **Table 5.**

The current study contrasts with [28], who conducted a study in Egypt on 50 chicken samples, in which *E. coli* O157 was detected in 3 samples. The current study agreed with a study conducted by[29] in USA on 2181 chicken meat using the traditional culturing method; none of the *E. coli* isolates was identified as STEC. Also, the current findings agree with [30], who conducted a study in Washington DC and failed to detect STEC in 59 chicken samples. Similarly,[31] didn't isolate STEC from 36 chicken samples in New Zealand, and [32] did not find STEC in 75 raw chickens in the Netherlands. On the other hand, this study contrasts with [33], who conducted a study in Iran on 422 chicken meat samples using culture technique and confirmed positive isolates using PCR assays. Results revealed confirmation of *E. coli* in 146 samples out of 422 tested samples. Among the *E. coli* positive samples, 31were from the enterohemorrhagic *E. coli* (EHEC)subgroup. All the positive samples for the EHEC subgroup had stx1 and eaeA andO157 most prevalent. Over and above, this study does not agree with [34] conducted a study in India on 500 chicken samples using multiplex polymerase chain reaction (m-PCR) in which only 11 samples were positive for the *eaeA* gene and absent for the *stx* genes. [29] Who conducted a study in Egypt on 50 chicken samples, in which *E. coli* O157 was detected in 4 samples. [35] in USA, was not in agreement with the current study as four STEC isolates were recovered from 33 chicken breast samples and qPCR was used for confirmation.

Beef meat is a source of high-quality protein and nutrients, may hinder disease-carrying pathogens such as *Salmonella, E.coli, Shigella, S.aureus,* and *L.monocytogenes*. *E.coli* are bacterial strains that could be found in beef and they can produce large quantities of a potent toxin that cause severe damage to the lining of the intestine. *E. coli* can colonize in the intestines of animals, which could contaminate muscle meat at slaughter. It can be easily destroyed by cooking [36]. The current study results clearly showed that seven out of the twenty beef samples that were investigated were identified as STEC, including 13 virulence genes. Whereas two samples hinder three virulence genes,*stx1*,*stx2*and *eaeA*, two samples are positive for two virulence genes only,*stx2* and *eaeA* and the three samples are positive for only*stx2* **(Table 4).** Additionally, out of the eighteen isolates obtained, ten were identified as *E. coli* O157 and two isolates *E. coli*O111 other six were identified as *E. coli* O26 **(Table 5).** Results match [36], who conducted a study in Argentina using PCR assay for serotyping and study of virulence genes of STEC isolated from 90 raw beef samples. Seventy beef samples were identified as STEC, 23 samples were identified as STEC O157, genotypedas*stx2*, and *eaeA.* Forty-seven samples were identified as STEC non-O157, genotypedas*stx1* and *stx2.*

Furthermore, this current study agrees with [37], who conducted a study in Iran using PCR assays to detect STECon820 raw beef, in which 238 (29.02%) samples were positive for the presence of *E. coli*. The isolates had more than one virulence genes*stx1*, *stx2* and *eaeA*. Similarly, this study goes in with [38], who investigated the presence of STEC in Sweden using real-time PCR on 177 samples, in which 27 STEC were isolated belonging to 14 different serogroups. STEC O157 was found in 2 samples. Over and above, this study agrees with a study conducted in Egypt by [28] on 50 beef samples in which *E. coli* O157 was detected in 3 samples. On the other hand, the current study goes in contrast with the results of a study done by [39] which demonstrated the inability to isolate the O157 serotype from minced meat, burger and luncheon, and [40] who conducted a study

in Brazil on 20 raw beef samples and failed to isolate *E. coli* O157 in any of the samples. Our team agrees with [41], who refers the variation in the prevalence of STEC to the type and source of samples tested and the testing methods in use.

The current study showed that beef had the highest prevalence of STEC. The presence of the *stx2* in all of STEC isolates (7) obtained from the seven positive samples is an exciting finding because *stx2* has been epidemiologically more associated with severe disease in humans than *stx1*[42]. Similarly,[43] indicated that the *stx2* genes were more prevalent than *stx1* genes among STEC strains isolated from cattle in Paraná State**.**[44] epidemiologic data suggest that isolates producing *stx2* alone caused more-severe neurologic symptoms than those producing both *stx1* and *stx2*, whereas those producing *stx1* alone induced only diarrhea. However, this contrasts with a study conducted by [45] in Egypt, which indicated that *stx1* predominated over *stx2* in STEC isolates from food products.

E. coli O157 and *E. coli* O26 were the most frequent serotype of the four isolates obtained from the three positive beef samples, as shown in **Table 5.** It was clearly shown that ten STEC strains belonged to O157 serogroup, eight to non-O157 serogroup, six to serotype O26 and two to serotype O111. These findings agree with [46], who found that *E. coli* O157:H7 is currently the most common serotype globally. Additionally, *E. coli* O26 is the most frequently isolated non-O157 Shiga-toxigenic *E. coli* (STEC) associated with human clinical illness[47]. In Europe, serogroups O157 and O26 were the most prevalent, representing 56 & 12% of the STEC isolates, respectively. However, in Italy, STEC O26 was the most commonly isolated serogroup in 2012, responsible for 40% of confirmed human cases, followed by STEC O157 & STEC O111, isolated from 33 and 12% of cases, respectively[48].

The current study indicates presumptive detection of STEC due to failure to recover STEC from some samples positive for stx genes. This might be due to very small numbers of STEC cells that could not be isolated on the selective agar and the presence of stx-carrying phages in the beef samples [49] or consumption of raw or undercooked may constitute a risk for STEC infection [54]. Also, as estimated by [55] that Shiga toxin-producing *Escherichia coli* (STEC) that cause severe disease predominantly carry the toxin gene variant *stx*2a. The failure to isolate STEC from the *stx*-positive samples may be due to the perturbation of high levels of background microflora, the loss of *stx* prophages during subculture, the presence of other bacteria carrying *stx*, low levels of STEC in the samples, addition of novobiocin to the enrichment medium or due to the presence of viable but non-culturable (VBNC) bacterial cells[49].

CONCLUSION

Ruminants, especially cattle, are the main reservoir for STEC and humans are infected by direct or indirect contact with the animals or their feaces or by consuming their edible products. Outbreaks are due to consumption of undercooked ground beef or beef products contaminated with STEC. The infectious dose of STEC is very low, for O157:H7 less than 50 organisms are enough to cause infection. STEC are heterogeneous, carrying various virulence genes, not all of which are equally pathogenic for humans. Thus, characterization of the virulence genes of STEC is vital for risk estimation.

The current study was designed to explore the prevalence and virulence characteristics of STEC bacteria isolated from 80raw materials and food (chicken, milk, beef and water) and to identify their serotypes using real-time polymerase chain reaction. Results obtained from this analysis are well qualified and controlled due to an accredited method. The proposed method has been validated and accredited according to ISO 13136:2012. This method has reproducible and reliable results with satisfactory detection limits and a short analysis time for the routine analysis of STEC in food. Furthermore, it enables the selective and simultaneous analysis of these virulence genes (*stx1, stx2, eaeA*) in different raw products. Based on the obtained results, pathogenic *E. coli* strains were mainly found in the one chicken sample and seven raw beef samples revealing 15 STEC virulence genes, including 24 serotypes. This is an interesting observation that should be considered for health intervention purposes all over Greater Cairo. Therefore, the recovery of STEC from raw chicken and beef samples is considered a significant concern. Furthermore, identifying potential sources of STEC strains could help establish control and prevention strategies.

Public health should take immediate actions to avoid any suspected outbreaks. This could be done through education to prevent transmission and exposure risks, identifying likely sources of exposure, mainly commercial or public authorities, identifying symptomatic contacts and arranging to test, conducting interventions if case exposed at or present while symptomatic in a childcare facility, congregating living, and finally providing standard precautions.

The observed results imply the need to take good care in the slaughter of cattle, the bacteriological analysis of food at the markets, and the techniques of disinfection and hygienic handling of foods, mainly raw minced beef. It is essential to keep food safe to prevent foodborne illness. Although consumer habits are critical to limiting foodborne disease by STEC, well-cooked beef is much safer, reducing the risk of HUS and other STEC-related diseases. It is highly recommended to follow all the rules and regulations stated by the world health organization to prevent the development of foodborne pathogens and prevent humans from acquiring pathogenic infections.

ACKNOWLEDGEMENT

I acknowledge the continuous support and comments received from QCAP.

CONFLICT OF INTEREST:

The authors declare no conflict of interest

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