Occurrence of shiga toxin-producing *Escherichia coli* in lactating cows and in contact workers in Egypt: serotypes, virulence genes and zoonotic significance

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Abstract: Shiga toxin-producing *Escherichia coli* (STEC) are emerging food–borne zoonotic pathogens associated with haemorrhagic colitis and haemolytic uremic syndrome in humans. This study investigated the occurrence of STEC serotypes, and prevalence of STEC isolates carrying virulence genes including shiga toxins (*stx*$_1$, *stx*$_2$); intimin (*eaeA*) and enterohaemolysin (*ehxA*) genes in lactating cows and in contact workers. Two hundred and forty samples of milk and cows' rectal swabs (120, each) and fifty stool swabs from in contact workers were collected from dairy farms at Sharkia Province, Egypt. These swabs were cultured on Eosin methylene blue agar. After biochemical and serological identifications, *E. coli* isolates were subjected to multiplex PCR. Seven enteropathogenic *E. coli* (EPEC) strains (O1, O2, O55, O86, O114, O119&O124); two enterohaemorrhagic *E. coli* (EHEC) strains (O26 &O111) and two enterotoxigenic *E. coli* (ETEC) strains were identified. The overall incidence of *E. coli* (19.1%) in milk showed a significant difference (P <0 .05) than that found in cows' rectal swabs (63.3%) and in contact workers' stool swabs (44%). The overall prevalence of milk STEC isolates possessing *stx*$_2$ (26.1%) clarified a significant difference (P <0 .05) than that obtained in *stx*$_2$ positive isolates (6.6%) from cows' rectal swabs. Moreover, human STEC isolates carrying *stx*$_2$ (22.7%) illustrated no significant difference. Of 23 isolates from milk, 3 (13%) STEC strains possessed *stx*$_1$, 6(26.1%) carried *stx*$_2$, 2(8.6%) harbored both *stx*$_1$ and *stx*$_2$ genes, and 4(17.3%) had *eaeA* or *ehxA* gene. The prevalence of STEC strains carrying *stx*$_1$, *stx*$_2$, *stx*$_1$+*stx*$_2$ and *eaeA* or *ehxA* in 76 cattle isolates was 7.8, 6.6, 5.2 and 7.8%, respectively. Out Of 22 isolates from dairy workers, 3(13.6%) STEC strains had *stx*$_1$, 5(22.7%) harbored *stx*$_2$, 1(4.5%) had both *stx*$_1$ and *stx*$_2$, 4(18.1%) carried *eaeA*, and 3(13.6%) possessed *ehxA* gene. The frequency distribution of virulence genes associated with STEC isolates was more evident in EHEC strains (O26 & O111 serogroups). This study emphasized that lactating cows are asymptomatic carriers of zoonotic STEC strains, and thus dairy workers should follow sound hygienic measures during milking and management of these animals to avoid public health hazards.

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1. Introduction

Shiga toxin- producing *Escherichia coli* (STEC) have been recognized as emerging food-borne zoonotic bacteria causing diarrhoea, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans (Pradel *et al*., 2008); with bovine feces being the main source for food contamination. Dairy cattle are implicated as a natural reservoir for non-O157 STEC strains because STEC serotypes are found as a part of the normal intestinal flora of lactating animals (Gyles, 2007; Bosilevac and Koohmaraie, 2011). These pathogens were excreted in faeces of dairy animals (Caprioli *et al*., 2005); then soiled teats and consequently contaminate raw milk during milking process (Hussein and Sakuma, 2005).

The evidence of zoonotic transmission of STEC was associated with consumption of unpasteurised milk and dairy products (Martin and Beutin, 2011; Farrokh *et al*., 2013).The STEC strains of animal origin (about 82%) belong to similar serotypes detected in humans, and 51% of these belong to serotypes related to human infection with HUS (Blanco *et al*., 2004a). Also, other infection routes may occur through human to human transmission, direct contact with carrier animals and indirect contact with contaminated environments(Keen *et al*., 2006).

In fact, non-O157 STEC pathogenesis is not fully understood (Bolton, 2011). As the capacity to induce illness depends on virulence factors that are required to assess the public health significance of emerging non-O157 strains. The STEC serotypes of human and bovine origins can elaborate virulence genes responsible for pathogenicity. These factors include shiga toxin type 1 gene (*stx*$_1$), shiga toxin type 2 gene (*stx*$_2$), intimin (*eaeA*) and enterohaemolysin (*hlyA*) (Paton and Paton, 1998). The severity of STEC infection is assessed to a large extent by the presence or absence of such factors because HUS progression is closely associated with the presence of shiga toxin genes (Hedican *et al*., 2009).

Multiplex PCR is an effective method in detection of specific virulence genes of STEC serotypes encoding shiga toxins 1 and 2, intimin and enterohaemolysin A, as reported in previous studies (Yu and Thong, 2009; Bai *et al*., 2010).With concern to public health, two human STEC outbreaks associated with raw milk consumption were attributed to O26 serotype in Austria (Allerberger *et al*., 2003); and to O80 and O145 strains in Germany (RKI, 2008). However, the prevalence and virulence factors of STEC isolates were determined in milk of mastitic cattle in Egypt (Osman *et al.*, 2012).Yet to our Knowledge; little literatures are available on the occurrence of STEC virulence genes in milk of healthy cattle and stool of in contact workers in Egypt. Thereby, the objectives of present study was to investigate the occurrence of STEC serotypes as well as to determine the frequency distribution of four virulence genes (*stx*$_1$, *stx*$_2$, *eaeA* and *ehxA*) in STEC isolates from milk, rectal swabs of lactating cows and stool swabs of in contact workers in Egypt to assess the pathogenicity and zoonotic importance.

2. Material and Methods

Sample collection and Preparation

A total of two hundred and forty samples of raw milk and cows' rectal swabs (120, each) collected from dairy farms at Sharkia Province, Egypt. Each rectal swab and milk sample was obtained from the same lactating cow. Milk samples were collected in clean, dry, sterile capped bottles. While, fifty stool swabs from in contact workers were obtained after having an informed consent. Each rectal and stool swab was individually immersed in sterile test tubes containing 9 ml buffered peptone water (BPW). One ml of each milk sample was aseptically homogenized with nine ml of BPW. All samples were transferred directly in an insulated ice box for analysis without delay at Zoonoses Laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Isolation of non-O157 *Escherichia coli* serogroups

The inoculated rectal, stool swabs and milk samples, into BPW, were incubated at 37°C for 18-24 hrs. One ml from the pre-enriched sample in BPW was transferred to 5 ml MacConkey broth and incubated at 37°C for 18-24 hrs. A loopful from the enrichment broth was streaked directly onto Eosin methylene blue (EMB) agar (Oxoid, CM69) then incubated at 37°C for 18-24 hrs (Ojo *et al*., 2010). All metallic green shinny colonies on the plates were purified on nutrient agar slants and incubated at 37°C for 18-24 hours for further identification.

Biochemical and serological identification

Suspected isolates of *E. coli* were biochemically identified(Barrow and Feltham, 1993).The serotyping of confirmed *E. coli* isolates was carried out by using rapid diagnostic *E. coli* monovalent and polyvalent antisera sets (DENKA SEIKEN Co., Japan) at Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt(Kok *et al*., 1996).

Genomic DNA extraction

The DNA pellet was extracted from *E. coli* culture of each isolate enriched in brain heart infusion broth using bacterial DNA extraction Kit (Spin-column) (BioTeke Corporation, Shanghai, China) according to the manufacturer instructions.

Primer design

Multiplex PCR was used to detect the frequency distribution of four virulence genes (*stx*$_1$, *stx*$_2$, *eaeA*& *ehxA*) in each *E. coli* isolate using specific oligonucleotide primers (Alpha DNA, Montreal, Quebec, Canada) as previously designed by Bandyopadhyay *et al*.(2011). Sequences of eight primers were used in this study as the following (stx1F; ATAAATCGCCATTCGTTGACTAC, stx1R; AGAACGCCCACTGAGATCATC, stx2F; GGCACTGTCTGAAACTGCTCC, stx2R; TCGCCAGTTATCTGACATTCTG, eaeAF; GACCCGGCACAAGCATAAGC, eaeAR; CCACCTGCAGCAACAAGAGG, ehxAF; GCATCATCAAGCGTACGTTCC and ehxAR; AATGAGCCAAGCTGGTTAAGCT). The predicted amplicon sizes were 180 bp for *stx*$_1$ gene, 255 bp for *stx*$_2$ factor, 384 bp for *eaeA* marker and 534 bp for *ehxA* gene.

Multiplex PCR

The PCR reaction was done in 25 µl reaction volume containing 12.5 µl of ready-made 2X power Taq PCR master mix (BioTeke Corporation); and 0.5 µM of each forward and reverse primers and 2 µl of DNA template. A positive control of STEC isolate (O26 serotype harboring *stx*$_1$ gene) was kindly supplied from Zoonoses Department, Faculty of Veterinary Medicine, Zagazig University. The reaction mixture without DNA was run in the thermal cycler, and was considered as a negative control.

The PCR condition for amplification was conducted according to Bandyopadhyay *et al*. (2011). Briefly, initial denaturation was performed at 95^{o}C for 5 min followed by 15 cycles of denaturation at 95^{o}C for 1min, annealing at 65^{o}C for 2 min and extension at 72^{o}C for 120s; and then 20 cycles of denaturation at 95^{o}C/1 min, annealing at 60^{o}C/2 min and extension at 72^{o}C/2 min. The final extension was carried out at 72^{o}C for 10 min. Amplification was done in a thermal cycler (Techno TC 3000, England). The PCR products were electrophoresed in ethidium bromide stained 1.5% agarose gel for 25 min at 120 V using agarose gel electrophoresis unit (Compact XS, Germany). The PCR amplicons in the gel were visualized by UV transilluminator (Biodoc analyze, Biometra,

Germany). The figures were captured by CCD camera mounted on Biodoc analyze.

Statistical analysis

The data were analyzed by Chi- square (IBM SPSS Statistics computer program, version 21) followed by a multiple Z-test to compare the column proportions in significant cases. *P* values < 0.05 were considered significant.

3. Results

In this study, we investigated the occurrence of *E. coli* serotypes and also the distribution of virulence genes associated with STEC isolates from milk, rectal swabs of lactating cows and stool swabs of in contact workers. From Table 1, seven enteropathogenic *E. coli* (EPEC) strains (O1, O2, O55, O86, O114, O119 &O124); two enterohaemorrhagic *E. coli* (EHEC) strains (O26 &O111) and two enterotoxigenic *E. coli* (ETEC) strains (O127&O128) were isolated in our study. The occurrence rate of *E. coli* serotypes in cows' milk (19.1%) was significantly different from those found in rectal swabs of lactating cows (63.3%) and also in dairy workers' stool swabs (44%) as shown in Table 1 (*P* <0.05). With regard to *E. coli* serotypes, the incidence rate of O111:K58 strains (EHEC) in milk (5%) showed a significant difference than that obtained from cows' rectal swabs (15%) as *P*-value was <0.05. While the occurrence of O111 serotype in humans' stool swabs (8%) illustrated no significant difference from that isolated from milk and rectal swabs of animals.

Also, the virulence factors associated with STEC isolates were detected in *E. coli* serotypes from different sources (milk, rectal swabs and stool swabs) by multiplex PCR (Figures 1, 2 & 3). Concerning the distribution of virulence markers (Table 2), only the overall incidence of STEC isolates harboring $stx_2$from milk samples (26.1%) revealed a significant difference than that strains positive for stx_2 and isolated from cows' rectal swabs (6.6%) as *P*-value was <0.05. Whereas, 22.7% of STEC isolates from farm workers positive for stx_2, exhibited no significant difference. The overall percentages of STEC isolates carrying stx_1 was 13, 7.8 and 13.6 % in milk, rectal swabs and stool swabs, respectively. On the other hand, the overall prevalence of STEC isolates carrying stx_1/stx_2 genes were 8.6% in milk, 5.2% in cows' rectal swabs and 4.5% in humans' stool swabs. As shown in Table 2, the prevalence rates of STEC isolates from milk, cows' rectal swabs & humans' stool swabs either harbored stx_1 or stx_1/stx_2 or *eaeA* or *ehxA* genes showed no significant differences at all. It is surprising; each of the four virulence genes was distributed in EHEC strains (O26&O111 serogroups) from milk samples and rectal swabs of cows with different percentages as declared in Table 3. Also, the STEC isolates belonged to O111 serotypes, from stool swabs of workers, contained each of the four virulence factors. However, the EPEC strains belonged to O1&O2 serotypes have no virulence gene.

4. Discussion

Nowadays, the involvement of shiga toxin-producing *Escherichia coli* in sporadic cases and disease outbreaks are presently increased. Infrequent human cases are attributed to ingestion of milk and dairy products. As ruminants are healthy carriers of STEC, most dairy products may provide these bacteria with favorable conditions for their growth. The contaminated milk and dairy products are a potential source of zoonotic STEC strains (Farrokh *et al*., 2013). Also, recurrent outbreaks of life-threatening human infections were attributed to STEC/EPEC contaminated milk and milk products (Martin and Beutin, 2011).

As shown in Table 1, the prevalence of *E. coli* in cows' milk showed a significant difference than that obtained from cows' rectal swabs (*P* <0.05). With respect to the incidence rate of *E. coli* (19.1%) in milk samples, lower percentage (13.3%) was cited by Glal *et al*. (2013)in Egypt. However, higher *E. coli* incidence rates of 63% in Sudan (Ali and Abdelgadir, 2011) and 38% in India (Thaker *et al*., 2012)were reported. Comparing the prevalence of *E. coli* (63.3%) in rectal swabs of cows, lower prevalence of 15.2% in Nigeria (Ojo *et al*., 2010) and 26.8% in Australia (Hornitzky *et al*., 2001) were recorded. The prevalence of *E. coli* in dairy workers illustrated a significant difference (*P* <0 .05) from that found in milk samples. This study revealed an infection rate for *E. coli* (44%) in dairy workers. This higher *E. coli* percentage may reflect poor personnel hygiene that followed during milking and in contact of such occupational workers to lactating cows.

As illustrated in Table 1, *E. coli* sero-groups from milk, cattle feces and human stool were isolated with different percentages and identified into EPEC strains (O119:K69; O86:K61; O2:K1: O1:K1; O55:K59; O124:K72; O114:K90) and EHEC serotypes (O26:K60; O111:K58) and ETEC strains (O127:K63; O128:K67). Similar *E. coli* serogroups (O26, O111 &O128) were isolated from cattle faeces (Ojo *et al*., 2010). Also our study was in concordant to relevant O111 serotype from milk and O119 serogroup from cattle faeces (El-Jakee *et al*., 2012). However, this study contradicted some *E. coli* serotypes isolated from raw milk sources in India (Thaker *et al*., 2012). Regarding *E. coli* serotypes from dairy workers; contrary finding reported *E. coli* serotype O164: K from human stool in Egypt (Osman *et al*., 2012). The current results suggested a potential public health threat of *E. coli* originating from milk and feces of dairy cattle.

Table 1. Occurrence of *Escherichia coli* serotypes and biotypes isolated from lactating cows and in contact workers, Sharkia, Egypt.

***Escherichia coli* serotypes**	***Escherichia coli* biotypes**	**Sources of *Escherichia coli* isolates**			***P*-values**
		Cows' milk Total examined = 120 **No.$^{\pi}$(%)**	**Cows' rectal swabs** Total examined=120 **No.$^{\pi}$ (%)**	**In contact workers' stool swabs** Total examined=50 **No.$^{\pi}$ (%)**	
O119:K69(B19)	EPEC$^{\triangle}$	5(4.1%)^{a}	11(9.1%)^{a}	6 (12%)^{a}	0.15
O86:K61(B7)	EPEC	3(2.5%)^{a}	0 (0%)$^{\neq}$	1 (1.3%)^{a}	0.231
O127:K63(B8)	ETEC$^{\infty}$	4 (3.3%)^{a}	5(4.1%)^{a}	2 (4%)^{a}	0.94
O26:K60(B6)	EHEC$^{€}$	2 (1.6%)^{a}	9(7.5%)^{a}	4 (8%)^{a}	0.08
O2:K1(H4)	EPEC	1(0.8%)^{a}	0 (0%)$^{\neq}$	0 (0%)$^{\neq}$	0.49
O111:K58(B9)	EHEC	6(5%)^{a}	18(15%)^{b}	4 (8%)^{a,b}	0.029*
O128:K67(B12)	ETEC	2(1.6%)^{a}	7(5.8%)^{a}	1 (2%)^{a}	0.17
O1:K1(H7)	EPEC	0 (0%)$^{\neq}$	0 (0%)$^{\neq}$	1 (1.3%)^{a}	0.09
O55:K59(B5)	EPEC	0 (0%)$^{\neq}$	0 (0%)$^{\neq}$	3 (6%)^{a}	0.12
O124:K72(B17)	EPEC	0 (0%)$^{\neq}$	14 (11.6%)^{a}	0 (0%)$^{\neq}$	<.000*
O114:K90	EPEC	0 (0%)$^{\neq}$	12 (10%)^{a}	0 (0%)$^{\neq}$	<.000*
Total		**23(19.1%)^{a}**	**76(63.3%)^{b}**	**22 (44%)^{b}**	<.000*

$^{\pi}$Number of *Escherichia coli* isolates.

$^{\triangle}$Enteropathogenic *Escherichia coli*.

$^{\infty}$Enterotoxigenic *Escherichia coli*.

$^{€}$Enterohaemorrhagic *Escherichia coli*.

* The Chi-square statistic is significant at $P<0.05$. Values in the same row having different superscripts were significantly different at $P<0.05$ in the two-sided test of equality for column proportions (using the Bonferroni correction).

$^{\neq}$This category is not used in comparisons because its column proportion is equal to zero.

With regard to the overall distribution of virulence markers associated with STEC strains from lactating cows and in contact workers (Table 2), statistical analysis clarified only a significant difference ($P < 0.05$) for prevalence of STEC isolates carrying stx_2 from milk samples and that from cows' rectal swabs but not from stool swabs of farm workers. Of 23 isolates from milk, multiplex PCR showed that 3 (13%) STEC strains possessed stx_1, 6 (26.1%) carried stx_2, and 2(8.6%) harbored both stx_1 and stx_2 genes. Otherwise; in comparing the occurrence of STEC isolates harboringstx_2 from milk of dairy cows, higher prevalence rates of 50, 93.7 and 75.7% were reported by Pradel *et al*. (2008); Stephan *et al*. (2008) and Bandyopadhyay *et al*. (2012), respectively. It is surprising, the frequency distribution of stx_2 gene associated with STEC isolates from milk showed higher percentages of 100 and 33.3% for O26 and O111 serogroups (Table 3 & Figure 1), respectively. Higher distribution of stx_2 positive isolates from milk may have possible public health hazards and zoonotic implications as the STEC strains that express stx_2 were linked to the development of HUS in humans (Islam *et al*., 2008).

Table 2. The overall distribution of virulence markers in STEC isolates from cows' milk, cows' rectal swabs and stool swabs of in contact workers, Egypt.

Virulence genes in STEC isolates	**Sources of *Escherichia coli* isolates (Total No.$^{¥}$)**			***P*-values**
	Milk (23)	**Rectal swabs (76)**	**Stool swabs (22)**	
	No. STEC isolates (%)	**No. STEC isolates (%)**	**No. STEC isolates (%)**	
stx$_1$	3(13%)^{a}	6(7.8%)^{a}	3(13.6%)^{a}	0.63
stx$_2$	6(26.1%)^{a}	5(6.6%)^{b}	5(22.7%)^{a,b}	0.019*
stx$_1$/stx$_2$	2 (8.6%)^{a}	4(5.2%)^{a}	1(4.5%)^{a}	0.80
eaeA	4(17.3%)^{a}	6 (7.8%)^{a}	4(18.1%)^{a}	0.26
ehxA	4(17.3%)^{a}	6 (7.8%)^{a}	3(13.6%)^{a}	0.38

$^{¥}$Total number of *Escherichia coli* isolates in relation to the isolation source.

*The Chi-square statistic is significant at $P<0.05$.Values in the same row having different superscripts were significantly different at $P< 0.05$ in the two-sided test of equality for column proportions (using the Bonferroni correction).

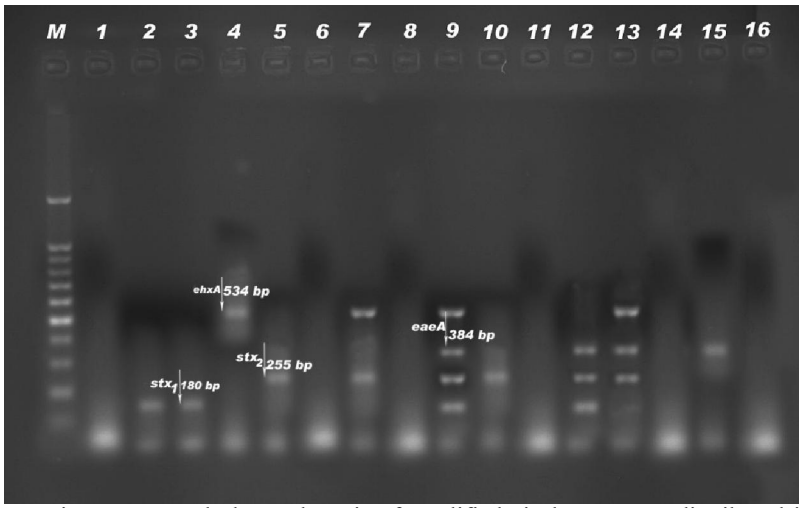

Figure 1. Representative agarose gel electrophoresis of amplified virulence genes distributed in STEC isolates from cows'milk by multiplex PCR.
M: Marker (100 bp); 1: negative control; 2: positive control for *stx*$_1$; 3: O119 serotype (+ve *stx*$_1$); 4: O119 (+ve *ehxA*); 5: O86 (+ve *stx*$_2$); 6:O68 (-ve); 7: O127(+ve *stx*$_2$ & *ehxA*); 8:O127 (-ve); 9: O26 (+ve *stx*$_1$, *stx*$_2$, *eaeA*& *ehxA*); 10: O26 (+ve *stx*$_2$); 11: O2(-ve); 12: O111(+ve *stx*$_1$, *stx*$_2$ &*eaeA*); 13: O111(+ve *stx*$_2$, *eaeA*&*ehxA*); 14: O111(-ve); 15: O128(+ve *eaeA*); 16: O128 (-ve).

The prevalence of milk STEC isolates carrying *stx*$_1$ gene disagreed with a lower percentage of 6.25% (Stephan *et al*., 2008) and also contrasted higher prevalence of 48.6% (Bandyopadhyay *et al*., 2012). The three *stx*$_1$ positive isolates from milk were belonged to O26, O111 and O119 serogroups (Table 3). In this study, each of milk STEC isolates carrying *eaeA* or *ehxA* had a similar prevalence rate (17.3%). Controversially, the prevalence of milk STEC isolates possessing *eaeA* (12%) and *ehxA* genes (37%) was cited by Pradel *et al*. (2008). Therefore, sound hygienic measures should be implemented at critical stages of milking to reduce the frequency of STEC contamination of milk derived from lactating cows.

With respect to STEC strains from cows' rectal swabs, occurrence of *stx*$_1$/*stx*$_2$ positive isolates (5.2%) in the present investigation (Table 2) was nearly corroborated with the finding of Hornitzky *et al*. (2005) from cattle feces in Australia. Of 76 cattle isolates in this study, 6(7.8%) possessed *stx*$_1$ and 5(6.6%) carried *stx*$_2$ alone. On the other hand, higher prevalence of STEC isolates carrying *stx*$_1$ (20%), *stx*$_2$ (54%), both *stx*$_1$ and *stx*$_2$ (26%) genes from cattle faeces were reported in Spain (Blanco *et al.,* 2004b). It was evident from Table 2, the overall distribution of *eaeA* or *ehxA* gene in cattle STEC isolates had a similar percentage of 7.8% (6 out of 76 isolates). However, *eaeA* was detected in 9(0.5%) of cattle STEC isolates in Australia (Hornitzky *et al*., 2002). Our study contradicted higher occurrence of positive *eaeA* STEC isolates (22.11%) from cattle faeces (Huasai *et al*., 2012). Also, this study disagreed with the finding of Blanco *et al*. (2004b); whereas higher percentages of cattle STEC isolates possessing *eaeA* and *ehxA* genes were found to be 63 and 29%, respectively. Of interest, the frequency distribution of virulence genes associated with cattle STEC isolates was more prominent in EHEC strains (O26&O111 serogroups) as illustrated in (Table 3& Figure 2). Thereby, our study evidenced that dairy cows are major natural reservoirs of STEC strains that constitute zoonotic risks, and the pathogens were excreted in their faeces as was previously supported by (Caprioli *et al*., 2005).

Concerning the distribution of virulence genes in 22 *E. coli* isolates from in contact workers (Table 2), the prevalence of STEC isolates carrying *stx*$_1$, *stx*$_2$, both *stx*$_1$ and *stx*$_2$, *eaeA* and *ehxA* was 13.6, 22.7, 4.5, 18.1 and 13.6%, respectively. On the contrary, higher occurrence of human STEC strains possessing *stx*$_1$ (60%) alone, both *stx*$_1$ and *stx*$_2$ (27%) were recorded (Hedican *et al*., 2009). Also prevalence of human STEC isolates in this study contrasted the findings of Eklund *et al*. (2001) and Blanco *et al*. (2004a). From Table 3& Figure 3, the frequency distribution of virulence markers linked with STEC isolates from dairy workers was more evident in EHEC strains (O26 and O111 serogroups). Collectively, the STEC isolates from in contact workers had similar virulence genes and serotypes to those identified from milk samples and rectal swabs of cows; and this evidenced the zoonotic transmission of STEC strains.

Table 3. Frequency distribution of virulence genes associated with STEC serogroups isolated from lactating cows and in contact workers, Sharkia, Egypt.

Source of isolates	*Escherichia coli* serotypes (number)	No. (%) of STEC isolates with:				
		stx$_1$	*stx*$_2$	*stx*$_1$/*stx*$_2$	*eaeA*	*ehxA*
Cows' milk	O119 (5)	1(20%)	0 (0%)	0 (0%)	0 (0%)	1(20%)
	O86 (3)	0(0%)	1(33.3%)	0(0%)	0(0%)	0(0%)
	O127 (4)	0(0%)	1(25%)	0(0%)	0(0%)	1(25%)
	O26 (2)	1(50%)	2(100%)	1(50%)	1(50%)	1(50%)
	O2 (1)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
	O111 (6)	1(16.6%)	2(33.3%)	1(16.6%)	2(33.3%)	1(16.6%)
	O128 (2)	0(0%)	0(0%)	0(0%)	1(50%)	0(0%)
All isolates	**(23)**	**3(13%)**	**6(26.1%)**	**2(8.6%)**	**4(17.3%)**	**4(17.3%)**
Cows' rectal swabs	O124 (14)	0(0%)	1(7.1%)	0(0%)	1(7.1%)	0 (0%)
	O111 (18)	3(16.7%)	1(5.5%)	1(5.5%)	2(11.1%)	2(11.1%)
	O114 (12)	0(0%)	0(0%)	1(8.3%)	0(0%)	0(0%)
	O26 (9)	1(11.1%)	3(22.2%)	1(11.1%)	1(11.1%)	2(22.2%)
	O128 (7)	0(0%)	0(0%)	0(0%)	1(14.3%)	1(14.3%)
	O119 (11)	1(9%)	0(0%)	0(0%)	0(0%)	1(9%)
	O127 (5)	1(20%)	0(0%)	1(20%)	1(20%)	0(0%)
All isolates	**(76)**	**6(7.8%)**	**5(6.6%)**	**4(5.2%)**	**6(7.8%)**	**6(7.8%)**
Stool swabs of in contact workers	O119 (6)	0(0%)	1(16.7)	0(0%)	0(0%)	0(0%)
	O26 (4)	1(25%)	2(50%)	1(25%)	0(0%)	1(25%)
	O55 (3)	0(0%)	0(0%)	0(0%)	1(33.3%)	0(0%)
	O111 (4)	1(25%)	1(25%)	0(0%)	1(25%)	2(50%)
	O128 (1)	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)
	O127 (2)	1(50 %)	0(0%)	0(0%)	1(50%)	0(0%)
	O86 (1)	0(0%)	0(0%)	0(0%)	1(100%)	0(0%)
	O1 (1)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
All isolates	**(22)**	**3(13.6%)**	**5(22.7%)**	**1(4.5%)**	**4(18.1%)**	**3(13.6%)**

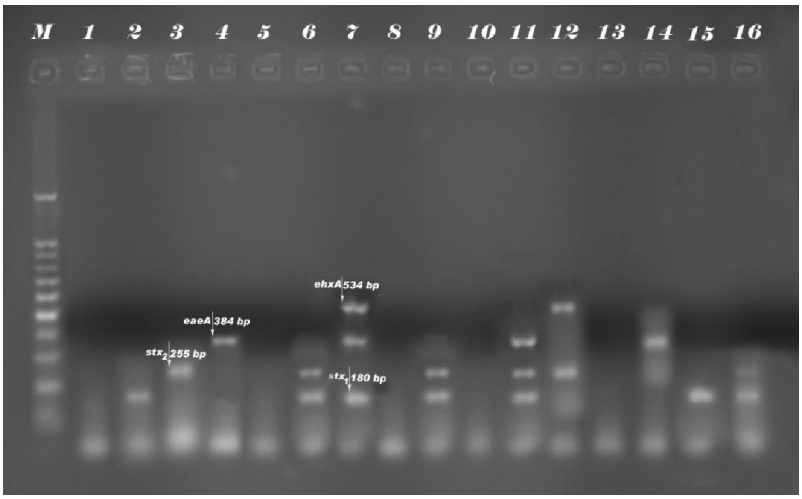

Figure 2. Representative agarose gel electrophoresis of amplified virulence markers associated with STEC isolates from cows' rectal swabs by multiplex PCR.
M: Marker (100 bp); 1: negative control; 2: positive control for *stx*$_1$;3:O124 serotype (+ve *stx*$_2$);4: O124 (+ve *eaeA*); 5: O124 (-ve); 6:O111 (+ve *stx*$_1$&*stx*$_2$); 7: O111(+ve *stx*$_1$, *eaeA* &*ehxA*);8:O111(-ve); 9: O114(+ve *stx*$_1$&*stx*$_2$); 10:O114(-ve); 11: O26(+ve *stx*$_1$, *stx*$_2$ & *eaeA*);12: O26(+ve *stx*$_2$ & *ehxA*); 13:O26(-ve); 14: O128(+ve *eaeA*); 15: O119(+ve *stx*$_1$);16: O127 (+ve *stx*$_1$& *stx*$_2$).

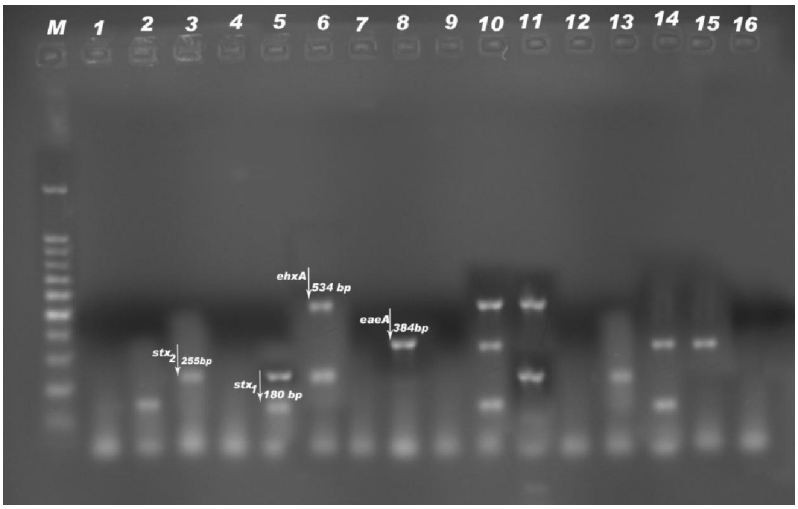

Figure3. Representative agarose gel electrophoresis of amplified virulence factors associated with STEC isolates from stool swabs of in contact workers by multiplex PCR.
M: Marker (100 bp); 1: negative control; 2: positive control for stx_1; 3:O119 serotype (+ve stx_2); 4: O119(-ve);5: O26 (+ve stx_1& stx_2); 6:O26(+ve stx_2 & *ehxA*); 7:O26(-ve); 8:O55(+ve *eaeA*); 9: O55(-ve); 10:O111(+ve stx_1, *eaeA*& *ehxA*); 11: O111(+ve stx_2 &*ehxA*); 12: O111(-ve); 13:O128(+ve stx_2); 14: O127(+ve stx_1& *eaeA*); 15: O86(+ve *eaeA)*; 16: O1(-ve).

4. Conclusion

Our study confirmed that milk and feces of lactating cows are important sources of zoonotic STEC with serotypes and virulence gene attributes that illustrate many similarities to those found in STEC isolates from in contact workers. The frequency distribution of virulence genes associated with STEC isolates was more evident in EHEC strains (O26& O111 serogroups). Thereby, dairy workers should follow the perfect hygienic measures during milking and management of lactating cows to reduce their infection with zoonotic STEC strains. Further studies are recommended on molecular subtyping of $stx_1$and stx_2 genes and other virulence markers associated with STEC isolates from dairy cattle.

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