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Full Length Research Paper

Molecular Epidemiology of *Escherichia coli* in Raw Cow's Milk: Prevalence and Genetic Determinants of Virulence

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Raw milk plays an important role in the survival and transport of pathogenic bacteria including *Escherichia coli* strains. This study was performed to determine the existence of *E. coli* in raw milk intended for human consumption and its associated virulence determinants. In this context, a total of 232 milk samples were obtained from different dairy shops located at Mansoura city and its surrounding villages. Milk samples were subjected for bacteriological and serological examination of *E. coli*. Furthermore, *E. coli* strains were tested for its haemolytic activity on blood agar plates. The recovered *E. coli* strains were also screened by Polymerase chain reaction (PCR) for the presence of enterotoxins including heat –labile (LT), heat- stable (ST) toxins and haemolysin (*hly*) genes. The recovery rate of *E. coli* was 14.65% (34/232). Among the recovered *E. coli* strains, 12 different *E. coli* serotypes were identified namely, O26:H11 (n=6), O111:H2 (n=5), O128:H2 (n=5), O91:H21 (n=4), O124 (n=3), O127:H6 (n=3), O103:H21 (n=2), O153 (n=1), O113:H4 (n=2), O6 (n=1), O121:H7 (n=1) and O146 (n=1). Regarding PCR results, 31(91.19%) *E. coli* strains harbored *STa* and seven strains carried *hly* gene (20.59%) while non *E. coli* isoates harbord *LT* gene. Conclusively, raw milk can be considered as serious source of pathogenic *E. coli,* therefore, proper management practices and effective control measures are recommended to improve milk hygiene and sanitation.

Key words: Raw milk, *Escherichia coli*, enterotoxin genes, haemolysin gene.

INTRODUCTION

Raw milk harbor variable microorganisms, considered as an important source of food borne pathogens because it is regarded as perfect media for microbial growth (Laba and Udosek, 2013). Consumption of raw milk may be associated with the occurrence of food-poisoning outbreaks (Christidis et al., 2016).The presence of different food borne pathogens in milk may be contributed to the fecal contamination during milking process (Rehman et al., 2014). *E. coli* is a normal inhabitant of the gastrointestinal tract of both man and animals. Most of *E. coli* strains are harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra

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Table 1. Sequences and cycling conditions of oligonucleotide primers.

Primer	Sequence	Amplified product	Reference	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177 bp	Piva et al. (2003)	94°C 5 min	94°C 30 s	60°C 1 min.	72°C 1 min	35	72°C 12 min
STa	GAAACAACATGACGGGAGGT GCACAGGCAGGATTACAACA	229 bp	Lee et al. (2008)	94°C 5 min	94°C 30 s.	57°C 30 s.	72°C 30 s	35	72°C 7 min
Lt	GGTTTCTGCGTTAGGTGGAA GGGACTTCGACCTGAAATGT	605 bp		94°C 5 min	94°C 30 sec	57°C 45 s	72°C 45 s	35	72°C 10 min

intestinal diseases in man (Croxen et al., 2013). Isolation of *E. coli* from milk represent a serious public health hazard because some strains of *E .coli* may be belongs to enteropathogenic or toxigenic or both groups which causes sever gastrointestinal disturbance (Thomas et al., 2017). Enterotoxigenic *E. coli* (ETEC) is one of the most common bacteria responsible for diarrhea in different parts of the world (Bagheri et al., 2014). Like other gastrointestinal infectious diseases, they are caused by lack of sanitation and most often contamination transfers from contaminated food or water (Walker et al., 2007; Marchou, 2013). There are two enterotoxins, Heat-stable toxin (ST) and Heat-labile toxin (LT). These two toxins are considered as the main virulence factors which influence the pathogenesis of ETEC strains (Kolenda et al., 2015; Sjöling et al., 2015). Alpha-hemolysin (HlyA) of *E. coli* is one of cytolytic pore-forming toxins (PFTs) produced by Gram-negative bacteria. *E. coli* HlyA lyses erythrocytes shows strong cytotoxic and cytolytic action against diversity of nucleated cells (Söderström et al., 2017). HlyA does not only kill and lyse cells but also affects target cells at sublytic concentrations. Haemolysin (*hlyA*) is produced mainly by extraintestinal pathogenic *E. coli* (ExPEC) strains and occasionally by ETEC, STEC and EPEC (Burgos and Beutin, 2010). Therefore, the main purpose of this study was to examine *E. coli* isolated from raw milk for the presence of enterotoxins including heat labile (LT) and heat stable (ST) toxins and haemolysin.

MATERIALS AND METHODS

Sampling

A total of 232 raw milk samples were collected randomly from different dairy shops, groceries and supermarkets in Mansoura city and its surrounding villages at Dakhalia Governorates, Egypt during the period from January to April, 2017. All samples were collected in sterile tubes and transported in an ice box to the laboratory as quick as possible for bacteriological examination with minimal of delay.

Isolation and identification of *E. coli*

All samples were immediately centrifuged and the sediment were streaked onto the surface of MacConkey's agar plates and incubated aerobically at 37°C for 24 h (Quinn et al., 2002). Lactose fermenting (Pink colored) colonies was sub-cultured on Eosin Methylene Blue (Oxoid) agar medium.

Colonies showing characteristic metallic green sheen on EMB agar were identified as *E. coli*. Presumptive *E. coli* colonies were subjected for gram staining and standard biochemical tests (Quinn et al., 2004). Additional identification of *E. coli* isolates was performed using commercial biochemical test kits (bioMerieux API, France).

Serological identification of *E. coli*

E. coli strains were transferred to Food Analysis Center, Faculty of Veterinary Medicine, Benha University for serological identification using rapid diagnostic *E. coli* antisera sets (Kok et al., 1996).

Haemolytic activity

E. coli isolates were cultured on blood agar containing 5% sheep blood, for detection of its haemolytic activity. Haemolysis was recorded after an overnight incubation at 37°C. A clear halo was defined as haemolysin positive (Brauner et al., 1990).

PCR assay for detection of enterotoxin genes (STa-LT) and haemolysin gene (*hly)*

Bacterial genomic DNA was extracted from *E. coli* isolates according to Ramadan et al. (2016). *E. coli* isolates were screened by Polymerase chain reaction (PCR) for the presence of enterotoxins (*Lt, STa*) and haemolysin (*hly*) genes. Oligonucleotide primers sequences and its amplicons sizes were described in Table1. Amplification reaction of PCR targeting haemolysin and enterotoxins was performed as previously described by Piva et al. (2003) and Lee et al. (2008), respectively (Table 1). Amplified DNA products for each gene were analyzed by 1.5% agarose gel electrophoresis in 1x TBE buffer stained with ethidium bromide visualized under UV transillumator.

RESULTS

In the present study, 34 (14.65%) *E. coli* strains have been recovered out of 232 examined milk samples. Among *E. coli* strains, twelve different *E. coli* serotypes were identified including, O26, O111, O128, O91, O124,

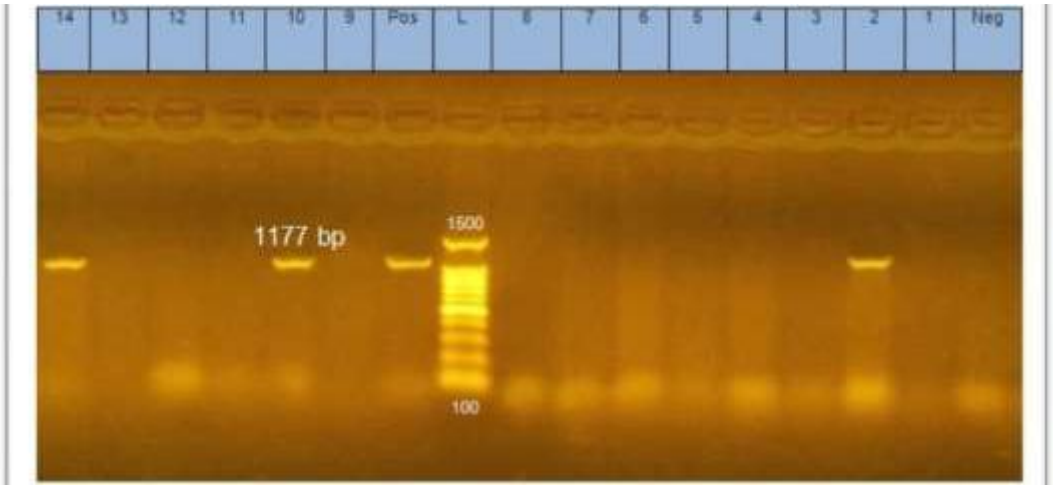

Figure 1. Agarose gel electrophoresis demonstrating amplification of *hly* gene at 1177 bp. Pos: Positive control, L: 100 bp DNA ladder, Neg: Negative control.

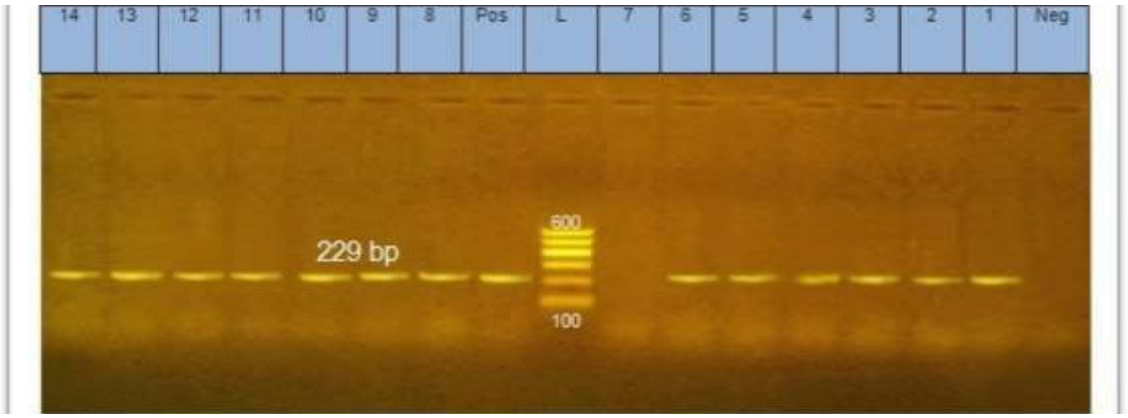

Figure 2. Agarose gel electrophoresis demonstrating amplification of STa gene at 229bp. Pos: Positive control, L: 100 bp DNA ladder, Neg: Negative control.

O127, O103, O113, O153, O6, O121 and O146 with a prevalence rate of 17.6, 14.7, 14.7, 11.7, 8.8, 8.8, 5.8, 5.8, 2.9, 2.9, 2.9 and 2.9% respectively.

E. coli isolates were tested for hemolytic activity on 5% sheep blood agar, 52.94% (18/ 34) of *E. coli* strains revealed different degrees of hemolysis. Based on the PCR results, 91.19% of *E. coli* isolates are potentially pathogenic, which carry one or more investigated virulence genes. From a total of 34 *E. coli* strains, 7(20.59%) strains carried *hly* gene (Figure 1), 31(91.17%) strains carried STa (Figure 2) while none *E. coli* isolates carried LT gene (Table 2).

DISCUSSION

Raw milk is a perfect medium that supports the growth and multiplication of *E. coli*. Consumption of such milk appeared as main threat to health concerns, especially for those people who still drink raw milk without heat treatment (Claeys et al., 2013). In the present study, *E.coli* was recovered with 14.65% prevalence rate. Similarly, *E. coli* has been isolated by several researchers from raw milk of cattle and buffaloes (Caine et al., 2014; Islam et al., 2008; Hossain et al., 2011). Compering to present results, a higher percentage of *E. coli* in milk was reported by Bandyopadhyay et al. (2012), Farzan et al. (2012), Mohd et al. (2013), Ali and Abdelgadir (2011) and Gwida and EL-Gohary (2013), who could isolate *E. coli* from raw milk in a percentage of 26.43,30.28, 33.96, 63 and 41.2% respectively. However, lower results were recorded by Kivaria et al. (2006) who detected *E. coli* in 6.3% of the examined raw milk samples.

In the present study, 12 different *E.coli* serotypes were identified; nearly the same serotypes were recovered

Table 2. Prevalence of serotypes, Enterotoxin (ST and LT) and haemolysin genes (hly) of *E. coli* isolated from raw milk.

Serotype (No)	Number (%)	hly gene (7)	Enterotoxin genes	
			LT	STa
O26:H11	6(6.71)	3	-	6
O111:H2	5(6.7.)	-	-	5
O91:H21	4 (667.)	-	-	4
O103:H21	2(875)	-	-	2
O113:H4	2(875)	-	-	-
O153	1(972)	1	-	1
O6	1(972)	1	-	1
O121:H7	1(2.9)	1	-	1
O146	1(2.9)	1	-	-
O124	3(8.8)	-	-	3
O127:H6	3(8.8)	-	-	3
O128:H2	5(14.7)	-	-	5
Total	34	7	-	31

from raw milk samples by Helmy et al. (2011) and Osman et al. (2012). Hemolysin is one of the important virulent factors in *E. coli*. In this study, 52.94% *E. coli* isolates revealed hemolysis on 5% sheep blood agar. Similarly, Farzan et al. (2012) reported that, one *E. coli* strain out of three isolates showed β-hemolytic activity on blood agar also, Lamey et al.(2013) found that 12.7% of isolated *E. coli* strains were hemolytic, Sayed (2014) found that one isolate (5.6%) out of 18 *E. coli* isolates had hemolytic activity. Concerning *hly* gene, 20.59% of *E. coli* harbored *hly* gene. A lower percentage was recorded by Ombarak et al.(2016), who identify *hly* gene in 2 (2.25%) isolates from karish cheese and one isolate (0.90%) from raw milk while, a higher percentages (42.85%) were reported by Osman et al. (2012). The presence of *E. coli* in milk especially enteropathogenic and/or toxigenic strains has a public health hazards which lead to sever gastrointestinal disturbance. Among *E. coli* isolates, 7(20.59%) strains carried *hly* gene, 31(91.17%) strains carried *STa* while, *LT* gene was not identified in all *E. coli* strains. Comparing to these results, Eid (2014) revealed that, only one strain were tested positive for *STa* gene out of eight E*. coli* isolates.

In Brazil, Paneto et al. (2007) studied the frequency of toxigenic *E*. *coli* in raw milk and cheese whereby, 1(2%) of *E*. *coli* isolates were ETEC. In Romania, Tabaran et al. (2017) analyzed 145 *E. coli* strains isolated from raw milk and traditional dairy cheeses, for the presence of *STa* and *STb*. In *LT,* none of the samples carries the *estI* gene, but 14 (9.7%) of the *E. coli* isolates were positive for both *eltA* and *estII*. Caine et al. (2014) examined 100 *E. coli* strain for the presence of enterotoxins which could identify enterotoxins in 4% of the total examined isolates.

Bonyadian et al. (2014) tested 120 isolates of *E. coli*, isolated from milk and unpasteurized cheeses which identified LT and STb in 2(1.66%) and 12(10.00%), respectively but could not identify *STa* gene. In this study, it was interesting that, all *E. coli* strains carry *hly* gene along with enterotoxin gene. These results suggest that food of animal origin represents a significant source of pathogenic *E. coli* strains.

Conclusion

The high contamination of milk with toxigenic *E. coli* represents a serious public health hazards which necessity high and strict preventive measures, to minimize the bacterial contamination within the food chain such as regular washing and sterilization of dairy equipment, utensils, milker's hands, animal udders as well as heat treatment of milk before distribution to consumers.

Significance statement

This study provided a data about the prevalence of *E. coli* in cow's raw milk, especially enterotoxigenic (ETEC) *E. coli*. These data is required for the establishment of food control systems which required the prevention and control of foodborne illnesses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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