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Molecular Detection of Enterotoxigenic *E. coli* in raw Milk and Milk Products

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A B S T R A C T

This study aimed to determine the incidence of *E. coli* in raw milk and cheese, in addition to isolate and identify Enterotoxigenic *E. coli*. The existence of heat stable toxin (STh) and heat labile toxin (LT) genes were determined in the isolated strains. This study included 350 samples of raw milk and cheese samples. Different media were used to isolate *E. coli* and different biochemical tests were used for identification. *E. coli* was detected in 61.4% of samples. They were tested for the presence of STh and LT genes by PCR. ETEC was detected in 3.7% of *E. coli* isolates. Only one strain from milk of street samples that found to harbor STh gene. Seven strains were detected in Kareish cheese including two strains harbor LT gene and five strains harbor STh gene.

Introduction

Escherichia coli is an inhabitant of the intestinal tract of animals and humans (Wetzel, 2005). Six different groups of pathogenic *E. coli* strains exist that harbor various virulence factors which enable them to cause diarrheal disease; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper *et al.*, 2004). A wide variety of food has been implicated as a vehicle of *E. coli* infection including milk. Most illness has been associated with eating undercooked and contaminated food also drinking unpasteurized milk (Altalhi and Hassan, 2009).

ETEC strains are emerging cause of food borne outbreaks, and have the ability to produce heat-stable and heat-labile enterotoxins. They are frequently associated with traveler's diarrhea and diarrhea in children (Wetzel, 2005). Diarrhea is watery and typically has an abrupt onset with an incubation period of 14 - 50 hours. Vomiting

is also a symptom but not fever. Mild to severe loss of fluids and electrolytes results in dehydration. More severe cases may require hospitalization (Wenneras and Erling, 2004; Qadri *et al.,* 2005).

In developing countries, ETEC can be isolated from both symptomatic and asymptomatic carriers, with significant mortality rates in children (Qadri *et al.,* 2005). In rural Egypt, a study showed that 84% of children under the age of 3 had at least one ETEC-related episode of diarrhea. The authors estimated that there were approximately 1.5 episodes/child/year related to ETEC (Rao *et al.,* 2003). The ETEC pathogenesis suggests that the organism colonize the small intestine by colonization factors, followed by the elaboration of heat-stable (ST) and/or heat-labile enterotoxin (LT). These virulence factors allow the organisms to readily colonize the small intestine and thus cause diarrhea (Qadri *et al.,* 2005). Coli surface antigen 6 CS6 is one of the most prevalent ETEC CS observed worldwide (Lapa *et al.,* 2008).

Since milk has almost neutral pH with high water content and a variety of nutrients, it represents an ideal substrate for microbial growth (Jay *et al.,* 2005). Milk contamination is usually associated with handling of milk by man and fecal contamination (Karns *et al.,* 2007). Presence of pathogenic bacteria in milk is of public health concern. Numbers of microorganisms including *Escherichia coli* can contaminate milk and milk products (Oliver *et al.,* 2005).

This work aimed to determine the incidence of *E. coli* in raw milk, Damietta and Kareish cheeses. Also, determine the incidence of enterotoxigenic *E. coli* by molecular detection of stable toxin (ST) and /or labile toxin (LT).

Materials and Methods

Collection of samples

A total of 350 random samples of raw milk and some milk products were collected from different sources in Assiut city. They were 200 samples of raw milk from farms, dairy shops and street vendors and 150 cheese samples (100 Damietta cheese and 50 Kareish cheese) from markets, vendors and super markets. Samples were transferred immediately in an ice box to the laboratory and stored at 4°C until examined within 24 hours.

Preparation of samples

Each milk sample was tested for detection of heat treatment by Storch test according to (Lampert, 1975) to exclude all heat treated milk samples.

Ten ml of milk sample was centrifuged for 20 minutes at 3000 r.p.m then the cream and supernatant were discarded. For cheese samples, 25 g of each sample was blended with 225 ml of nutrient broth (Difco) for 2 min. using a Stomacher lab blender, and then samples were incubated at 37°C for 24 h (Read *et al.,* 1990).

Enrichment of samples (Quinto and Cepeda, 1997)

One ml of prepared milk and cheese samples was mixed with 9 ml MacConkey's broth (Oxoid, UK) and incubated for 24 h at 37°C.

Selective plating (Cheesbrough, 2004)

A loopful of the incubated broth was streaked on MacConkey's agar and E.M.B. agar media. Plates were aerobically incubated at 37°C for 24 h.

Identification of *E. coli* isolates

Gram stained films of suspected *E. coli* colonies showing Gram negative, nonspore forming bacilli. Biochemical tests were used to confirm *E. coli* isolates. All *E. coli* isolates were positive for catalase, indole, and methyl red and negative for oxidase, urease, citrate and Voges-Proskauer. Sugar fermentation using triple sugar iron indicated acid slant and butt and gas production *(Collee et al., 1996).*

Identification of *ETEC* by PCR for detection of ST, LT genes

DNA extraction (Hassan and Elmalt, 2008)

Bacterial DNA was extracted following cultivation in trypticase soy agar (TSA-Scharlau, Spain.) at 37°C. A colony was suspended in 100 µl sterile distilled water and the suspension was boiled for 10 mins. Then centrifugation was done and the supernatant was used as a template for PCR and stored at - 20°C until use.

DNA amplification

STh and LT genes were amplified separately using primers described previously (Vidal *et al.,* 2009 and Nada *et al.,* 2010). Primers were purchased from Invitrogen, USA. The used primers sequences are described in Table 1.

Table.1 PCR was carried out in 50 µl reaction volume using master mix (Koma Biotech Inc., Korea)

Gene	Primer sequence 5′–3′	Product Band size (bp)
LT	sense-CATAATGAGTACTTCGATAGAGGAAC-	402
	antisense-GAAACCTGCTAATCTGTAACCATCC-	
STh	sense-TTCTTTCTGTATTGTCTTTTTCACC-	193
	antisense-TAATAGCACCCGGTACAAGCAG-	

PCR thermal cycler (Bio Rad T100, USA) was used with initial denaturation step at 95°C for 2 min., followed by 30 cycles of 95°C for 1 min., annealing temperature of 58°C for 30 sec. and elongation for 1 min. at 72°C and a final extension step at 72°C for 10 min. Ten µl from each PCR product were examined by electrophoresis in 1.5% agarose gel stained with ethidium bromide under U.V light.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0. Data was presented as numbers and percentages. Chi-square test was used to compare qualitative variables between groups. P-value < 0.05 was considered statistically significant.

Results and Discussion

Enterotoxigenic *Escherichia coli* (ETEC) is causing approximately 280-400 million diarrheal episodes in children under the age of five every year. It has been recognized as the most common cause of infectious diarrhea in infants and young children in developing countries (Wennerås and Erling, 2004). Also, the elderly are also susceptible to ETEC infections requiring hospitalization (Faruque *et al.,* 2004). The infection is caused by ingestion of contaminated food and water. Food can be contaminated by infected food handlers or asymptomatic carriers or when using untreated water (Croxen *et al.,* 2013).

Results recorded in Table 2 revealed that *E. coli* was isolated from 62% of total raw milk samples; 59% of dairy farms, 62% of dairy shops and 68% of street vendors. Lower results were mentioned by Kumar and Prasad (2010) who found that the highest contamination was recorded in milk from vendors (26%) followed by dairy farm (20%). In addition, lower results were reported by Bali *et al.,* (2013) (32.5%), Rashid *et al.,* (2013) (33.96%), Virpari *et al.,* (2013) (52%) and El nahas *et al.,* (2015) (55%). Compatible results were reported by, Ali and Abdelgadir

(2011) (63%) and Chye *et al.*, (2004) (65%), but higher results was found by Hassan and Elmalt, (2008) (76.0%) and Ibrahim *et al.*, (2015a) (100%).

High incidences of *E. coli* in the examined raw milk samples indicated neglected sanitary control in handling of milk and possibly fecal contamination. Microorganisms may gain entry into raw milk from the dairy animals experiencing subclinical or clinical mastitis, or from fecal contamination, particularly around the teats, and from the farm environment particularly the water source and utensils used for the storage of milk on the farm or during transportation (Ombarak and Elbagory, 2015).

Regarding the results in Table 3, *E. coli* was isolated from 60.7% of cheese samples; 36% of Damietta cheese and 73% of Kareish cheese. Many investigators mentioned lower incidence of *E. coli* in Kareish cheese as Virpari *et al.*, (2013) (28%) and El nahas *et al.*, (2015) (50%). Dissimilar higher results were reported by Paneto *et al.*, (2007) (96%), Najand and Ghanbarpour (2007) (98.7%) and Ibrahim *et al.*, (2015a) (100%).

Concerning Damietta cheese, rare research was done. However, lower results were reported by Ahmed, (2012) (30.48%) and Sharaf *et al.*, (2014) (7%). But higher results were reported by Ibrahim *et al.*, (2015a) (80%) and Ibrahim *et al.*, (2015b) (40%).

E. coli was recovered from 61.4% of the total examined milk and cheese samples (Table 4). Milk can be easily contaminated by infected food handlers who practice poor personal hygiene or by water containing human discharge. Therefore, water must be safe and practically free from pathogens. Also contamination of milk products is largely due to processing, handling, and unhygienic conditions and detection of *E. coli* in milk often reflects faecal contamination (El nahas *et al.*, 2015). Contamination of cheese with microorganisms may originate from many sources. Such sources during cheese production might be: starter culture, brine, floor and packaging material, cheese vat, cheese cloth and curd cutting knife, cold room and production room air (Sharaf *et al.*, 2014).

Table.2 Incidence of *E. coli* in raw milk samples

Examined raw milk samples	No. of examined samples	Positive samples		P value
		No.	%	
Dairy farms	100	59	59	0.574*
Dairy shops	50	31	62	
Street vendors	50	34	68	
Total	**200**	**124**	**62**	

*: No statistically significant difference (p>0.05)

Table.3 Incidence of *E. coli* in cheese samples

Examined cheese samples	No. of examined samples	Positive samples		P value
		No.	%	
Damietta cheese	50	18	36	0.001*
Kareish cheese	100	73	73	
Total	**150**	**91**	**60.7**	

* Statistically significant difference (p<0.01)

Table.4 Incidence of *E. coli* in raw milk, Damietta and Kareish cheese

Examined cheese samples	No. of examined samples	Positive samples	
		No.	%
Raw milk	200	124	62
Damietta cheese	50	18	36
Kareish cheese	100	73	73
Total	**350**	**215**	**61.4**

Table.5 Detection of STh gene in *E. coli* isolates

Examined samples		No. of positive *E. coli* samples	STh	
			No.	%
Raw milk	**Dairy farms**	59	-	-
	Dairy shops	31	-	-
	Street vendors	34	1	3.8
	Total	124	1	0.9
Cheese	**Damietta cheese**	18	-	-
	Kareish cheese	73	5	6.8
	Total	91	5	5.5
Total		**215**	**6**	**2.8**

Table.6 Detection of LT gene in *E. coli* isolates

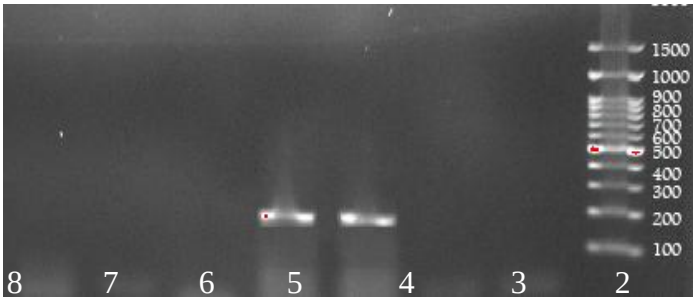
Examined samples		No. of positive *E. coli* samples	LT	
			No.	%
Raw milk	**Dairy farms**	59	-	-
	Dairy shops	31	-	-
	Street vendors	34	-	-
	Total	124	-	-
Cheese	**Damietta cheese**	18	-	-
	Kareish cheese	73	2	2.7
	Total	91	2	2.2
Total		**215**	**2**	**0.93**

Table.7 Incidence of ETEC among *E. coli* isolates

Examined raw milk samples	No. of positive *E. coli* samples	Total ETEC		P value
		No.	%	
Raw milk	124	1	0.9	0.028*
Damietta cheese	18	-	-	
Kareish cheese	73	7	9.6	
Total	**215**	**8**	**3.7**	

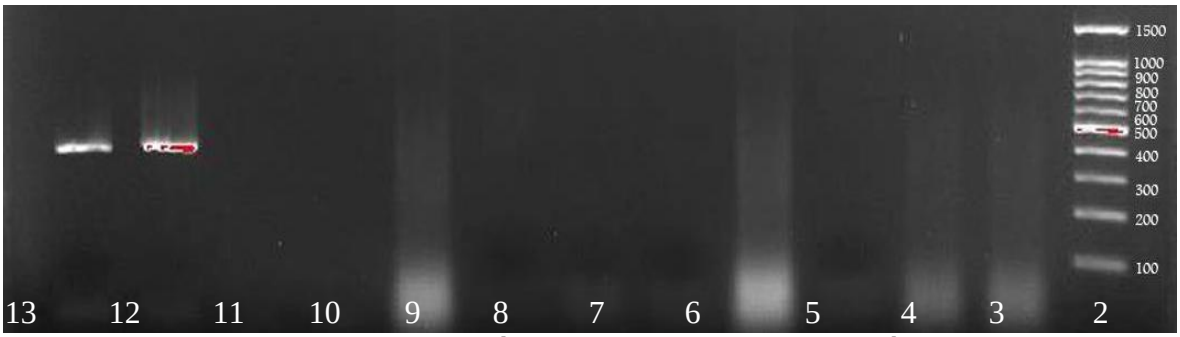
*****Statistically significant difference ($p<0.05$)

Fig.1 Agarose gel electrophoresis of amplified STh gene in positive *E.coli* (193 bp)



Lane 1: DNA marker, Lane 4, 5: positive STh *E. coli*

Fig.2 Agarose gel electrophoresis of amplified LT gene in positive *E.coli* (402 bp)



Lane 1: DNA marker, Lane 12 &13: positive LT *E. coli.*

Results clearly indicated that microbial quality and safety of raw milk, Damietta and Kareish cheese was unsafe. The presence of fecal indicator organism not only indicates the poor hygiene but also may be pathogenic itself.

ETEC strains are classified based on their expression of enterotoxins either heat labile toxin (LT), heat stable toxin (ST) or both (Nicklasson, 2008). STh has been reported to cause diarrhea in children as well as in adult travelers to different geographical areas (Bölin *et al.*, 2006). It is important to note that the levels of production of LT vary among different strains. Therefore, the regulation of LT production may occur differently in individual ETEC isolates (Mudrak and Kuehn, 2010). ETEC detection is reliant on detection of the enterotoxins LT and/or ST. Detection of both toxins is done by PCR based techniques. The PCR targets include the genes encoding LT and ST (O'Sullivan *et al.*, 2007).

ETEC were detected in 3.7 % of all milk and cheese samples. Only one isolate of *E. coli* from raw milk harbored STh gene so the incidence of ETEC in milk was 0.9 %. Two *E. coli* isolates of Kareish cheese had LT gene, while five had STh gene only, so ETEC were 9.6% of *E. coli* isolates.

Compatible results to the present work was found by many investigators as Bonyadian *et al.*, (2014) who reported 1.66% LT and STb in milk and cheese but none of the strains harbored the STa gene. Also El nahas *et al.*, (2015) reported 27.27% ETEC in milk samples and 15% in Kareish cheese. There was a significant difference between percentages of ETEC in raw milk, Damietta and Kareish cheese as shown in Table 7. ETEC were highly detected in Kareish cheese

then raw milk but was not detected in Damietta cheese.

STh gene was detected in one *E. coli* isolate of raw milk samples that collected from street vendors also, it was detected in five *E. coli* isolates from Kareish cheese but it was not detected in Damietta cheese (Figure 1). According to the PCR of STh gene, it was found in 3.8% of the isolates from street vendors raw milk samples that represented 0.9 % of the total raw milk samples. In Kareish cheese, STh was detected in 6.8 % of *E. coli* isolates but was not detected in Damietta cheese (Table 5). It is obvious that Kareish cheese represented a high level of contamination, hence a large source of infection. Results recorded in Table 6 and Figure 2 showed that LT gene was not detected in any of the isolates from raw milk samples and Damietta cheese but it was detected in two isolates (2.7 %) from Kareish cheese.

The proportions of ST-only, ST/LT, and LT-only strains vary between different studies and geographical areas. Roughly one third of all ETEC strains isolated globally have been reported to be ST- only strains, one third ST/LT and one third LT-only strains (Qadri *et al.*, 2005). In other studies the ST-only strains have been reported to constitute up to 50% of the strains. ST-positive ETEC strains are commonly associated with diarrhea and found to be major contributor to infantile diarrhea and associated with an increased risk of death (Qadri *et al.*, 2007).

This study indicated high contamination of milk and cheese especially Kareish cheese that is considered a public health threat. It is very important to apply standard sanitary measures to reduce contamination and infection and aware people around the importance of hygienic condition. Also, it is necessary to apply strict hygienic measures during milking and cheese production to minimize the contamination with such pathogen.

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