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Prevalence of *Escherichia Coli* in Some Selected Foods and Children Stools with Special Reference to Molecular Characterization of Enterohemorrhagic Strain

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Abstract: *Escherichia coli* is one of the most important etiologic agents of childhood diarrhea that represents a major public health problem in developing countries and now is being recognized as emerging entero-pathogens in the well developed countries. Among the Diarrhegenic *Escherichia Coli* (DEC) this work was focused on the Enterohemorrhagic *Escherichia Coli* (EHEC) that produce Shigatoxins (Stxs). The infection is mainly transmitted through food of bovine origin such as beef and dairy products that has been often associated with outbreaks which ranged from mild diarrhea to the life-threatening hemolytic uremic syndrome. The aim of this study was to evaluate the incidence of potentially virulent STEC isolates from minced meat, some selected dairy products and from children clinical cases. Out of 360 samples, a total of 115 *E.coli* isolates were recovered as following 35 (30.43%) isolate from meat, 21 (18.26%) from raw milk, 31 (26.96%) from cheese and 28 (24.35%) from children stool. The identification were based on Microscopical examination, Biochemical identification and Serotyping. Twenty eight isolates were identified serologically. O26:K60 serotype 6 (21.4%) was found to be the most prevalent serotype. Screening for virulence genes (Stx_1, Stx_2, *eaeA*, *hly*A) was done using multiplex PCR, which revealed detection of the target genes in 10 out of 115 (8.7%) examined samples. About 7 (70%) samples possessed *eaeA* gene alone; while 2 (20%) samples contained Stx_2 gene. Both Stx_1 and *eaeA* genes were detected in 1(10%) sample only.

Keywords: DEC, EHEC, Shigatoxins (Stxs), Childhood Diarrhea

Introduction

Escherichia coli is the predominant nonpathogenic facultative flora of the human intestine (Nataro and Kaper, 1998). Some *E. coli* strains, however, are the most important etiologic agent of childhood diarrhea and represent a major public health problem in developing countries (Toma *et al*., 2003). Among the *E. coli* causing intestinal diseases, there are six well-described pathotypes: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Stenutz *et al*., 2006).

The infection is mainly food-borne, but it can also be acquired by person-to-person spread or direct contact with animals. Ruminants and in particular cattle, are considered to be the principal reservoir of *E. coli*, so food of bovine origin such as beef and dairy products has often been associated with outbreaks (Perelle *et al*., 2007). Moreover, an increasing number of episodes have been associated with consumption of fruits and vegetables fertilized with ruminants' manure or contaminated during harvesting or processing (Conedera *et al*., 2007).

Among the six recognized diarrhegenic categories of *E. coli*, ETEC is the most common, particularly in the developing countries (Qadri *et al*., 2005). EHEC is the most important recently emerged group of food-borne pathogens. It can cause severe gastrointestinal disease, including fatal infections and is being detected more frequently worldwide. EHEC strains not only produce

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potent cytotoxins (verotoxins) but have also acquired the ability to adhere to the intestinal mucosa in an intimate fashion (Fagan *et al*., 1999). Specific virulence factors such as enterotoxins and colonization factors differentiate ETEC from other categories of diarrheagenic *E. coli* (Qadri *et al*., 2005). Many assays for the detection of diarrheagenic *E. coli* are available, such as biochemical reactions, serotyping, phenotypic assays based on virulence characteristics and molecular detection methods (Nataro and Kaper, 1998; Nguyen *et al*., 2005). Among these, Polymerase Chain Reaction (PCR) is one of the molecular biology-based detection methods, is a commonly used method that gives rapid, reliable results and that also has a high sensitivity and a high specificity (Nguyen *et al*., 2005).

Recently various multiplex PCR methods have been developed for the simultaneous detection of several pathogenic genes in one PCR reaction. These methods showed high sensitivity and specificity for identification of human diarreheagenic *E. coli* (Arif and Salih, 2010).

The aim of this study was to evaluate the prevalence of *E. coli* in collected food of animal origin such as minced meat, raw milk, kareesh cheese and in fecal samples of clinical cases of children and to investigate the characteristics and efficacy of multiplex PCR to simultaneously detect diarrheagenic *E. coli*.

Materials and Methods

Collection and Sampling Process

Three hundred samples of minced meat, fresh raw milk and Kareesh cheese (one hundred of each) are randomly collected from butcheries, supermarkets, dairy stores and milk vendors. In addition, 60 random diarrheal stool samples were collected from different hospitals and private laboratories of children clinical cases. All samples were collected from different localities in Ismailia Governorate; the samples were transported to the laboratory without delay in a temperature-regulated ice box to be examined.

Enrichment and Recovery of E.coli (ISO 16654:2001; De Boer and Heuvelink, 2000)

About 25 g. or mL of examined samples were added to 225 mL of modified Tryptone Soya Broth ([Difco) supplemented with novobiocin solution (20 mg L^{-1}). The samples were placed in individual bags homogenized in a stomacher for 2 min and incubated for 24 h at 37°C. Each recovered sample was streaked onto modified Sorbitol MacConkey agar and incubated at 37°C for 24 h. Both of Cexifime and potassium tellurite were added to the SMAC to increase selectivity especially for heavily contaminated samples.

In parallel, a loopful from the incubated broth was streaked into Eosin Methylene Blue Agar. The inoculated plates were incubated at 37°C for 24 hr. Typical colonies of *E. coli* appear greenish, metallic and with dark purple center. Suspected colonies were purified and sub-cultured onto nutrient agar slopes and incubated at 37°C for 24 hr, then stored until further investigation was carried out.

Confirmation of the Isolates

Were carried out according to Edwards and Ewing's Identification of Enterobacteriaceae (Farmer *et al*., 1985).

Serotyping

The confirmed *E. coli* isolated strains were serological identified by Central Laboratories of Ministry of Health-Bacteriology Department-Cairo.

Antimicrobial Susceptibility Testing (CLSI, 2011)

All identified serotypes were tested for determination of their antimicrobial profiles. Susceptibility to antimicrobial agents was determined by the standardized disk diffusion assay on Mueller-Hinton agar (Difco, USA) with 8 different commercial antimicrobial susceptibility disks (Bioanalyse, Turkey) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011) guidelines. The antimicrobial tested and their corresponding disk concentrations were as follows: Erythromycin (15 µg); Cefatriaxone (30 µg); Chloramphenicol (30 µg); Trimethoprim/Sulphamethoxazole(1.25-23.75 µg); Ciprofloxacin (5 µg); Azithromycin (15 µg); Amikacin (30 µg); Streptomycin (10 µg). Isolates resistant to 3 or more different classes of antimicrobial agent were defined as multi-drug resistant.

DNA Extraction

DNAs were extracted from reference strain that obtained from the Central Laboratories of the Ministry of Health Bacteriology Department- Cairo and isolated strains by the method described by (Yokoyama, 1993; Toma *et al*., 2003). The reference and isolated strains were cultured in 2 mL of Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking. Thirty-six microliters of broth culture was added to 4 µL of 10x Tris-EDTA buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.3) and 60 µL of 2x proteinase K buffer (100 mM KCl, 20 mM Tris-HCl, 5 mM MgCl2, 1% Tween 20, 800 µg of proteinase K/mL, pH 8.3). After incubation for 90 min at 56°C and 10 min at 95°C, the sample was centrifuged at 10,000 × g for 1 min and the supernatant was used as DNA template (Fig. 1). The DNA quantity and purity were assessed spectrophotometrically at 260-280 nm, which was determined with NanoDrop ND-1000 full spectrum UV-Vis spectrophotometer (USA).

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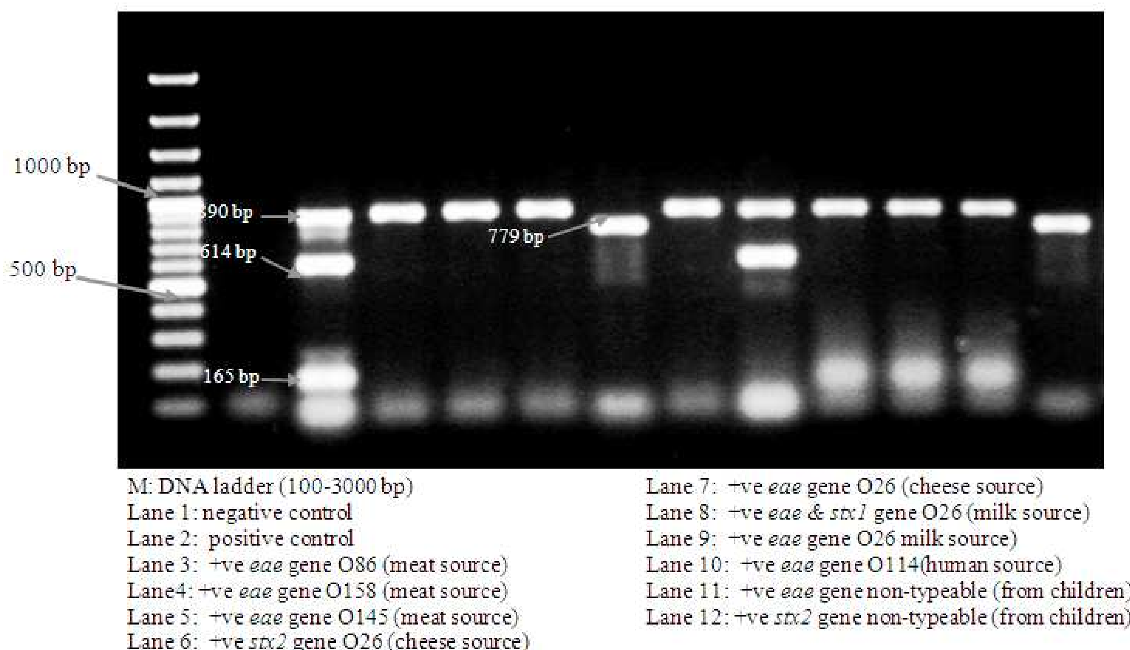

Fig. 1. DNA ladder

Table 1. Incidence of isolated *E. coli* from examined samples

Sample type Isolates	Minced meat (n = 100)	Raw milk (n = 100)	Kareesh cheese (n = 60)	Children stools (n = 100)	N = 360
	35 (30.43%)	21 (18.26%)	31 (26.96%)	28 (24.35%)	115(31.94%)

Molecular Characterization (Fagan et al., 1999)

Multiplex PCR for detection of Stx_1, Stx_2, eaeA and EHEC hlyA gene sequences was performed with an eppendorf MasterCycler Gradient (Eppendorf-Netheler-Hamburg, Germany). Oligonucleotide primers were manufactured commercially (Metabion International AG, Germany). Primers and the predicted lengths of PCR amplification products are listed in Table 1.

PCR assays were carried out in a 50 µL volume containing 2 µL of nucleic acid template prepared from isolated cultures (approximately 60 ng of DNA) or 1 µL (approximately 30 ng) of nucleic acid template prepared by using reference *E. coli* O157:H7 isolate which obtained from the Central Laboratories of the Ministry of Health Bacteriology Department- Cairo, 25 µL of Master Mix (Fermentas, USA). Temperature conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s and 72°C for 90 s. The final cycle was followed by 72°C incubation for 5 min (Fagan *et al*., 1999).

Ten microliters of PCR products were then electrophoresed on a 2% Agarose gel (Bioshop, Canada), stained with ethidium bromide (Sigma, USA) and visualized by UV transillumination. The buffer in the electrophoresis chamber and in the agarose gel was 1X TAE buffer. The amplicon was imaged with a gel documentation system LTF (Labortechnik, Germany).

Results

Percentage of isolated *E. coli* strains: A total of 115 *E. coli* strains were isolated regarding to the culture media and were confirmed by biochemical tests. About 35 (30.43%) strains were isolated from minced meat, 21 (18.26%); raw milk, 31 (26.96%); Kareesh cheese samples and 28 (24.35%) from the stool samples.

Serotypes Detection

Serogrouping was performed for all isolated *E. coli* strains. Only 28 (24.35%) strains were serologically identified, while 87 strains (75.65%) were non-typeable. Among the serological identified strains, EHEC were the most common type [8 isolates (6.95%)].

Table 2 the results of serotyping.

Antimicrobial Susceptibility Testing

All Identified serotypes were susceptible to different 8 antimicrobial agents. The results obtained in this study for each tested antimicrobial agent, have been reported according to (CLSI, 2011). The strains were classified as Sensitive (s), Intermediate (I) or Resistant (R) based on the inhibition zone diameter.

Table 2. Results of Serological identification of *E. coli* isolates from the examined samples

Samples	No of isolates	O-typable	O-untypable (OUT)
Raw minced meat	35	9 (25.71 %)	26 (74.29%)
Raw fresh milk	21	5 (23.80%)	16 (76.19%)
Kareesh cheese	31	9 (29.03%)	22 (70.97 %)
Stool samples	28	5 (23.80%)	23 (82.14 %)
Total	115	28 (24.34%)	87 (75.65 %)

Table 3. Incidence of identified *E. coli* serotypes according to samples sources and strain character

Strain Character	Serotype	Minced Meat (35 isolated strains)	Raw Milk (21isolated strains)	Kareesh Cheese (31 isolated strains)	Diarrheal Stools (28 isolated strains)	Total (115)
EPEC	O86:K61 (B7)	2 (5.71%)	-	-	-	10 (8.7 %)
	O124:K72	2 (5.71%)	2 (9.52 %)	-	-	
	O158:K-	1(2.86%)	-	-	-	
	O114:K90	-	-	-	3 (10.71 %)	
EHEC	O145:K-	1 (2.86%)	-	1 (3.23 %)	-	8 (6.95 %)
	O26:K60		3 (14.29%)	3 (9.67 %)	-	
ETEC	O25:K11	3 (8.57%)	-	-	-	5 (4.35 %)
	O115:K-:	-	-	1 (3.23 %)	-	
	O8:K-:	-	-	1 (3.23 %)	-	
EAEC	O44:K74	-	-	3 (9.67 %)	-	3 (2.61 %)
EIEC	O124:K86	-	-	-	2(7.15%)	2(1.74%)

Serological identification:

All *E. coli* isolates were subjected to serological identification using 9 polyvalent antisera then we applied monovalent antisera to positive reactions

Table 4. Percentage of antibiotic resistance of O-typable *E. coli* (n = 28)

Antimicrobial agent	Susceptible	Intermediate	Resistant	% Resistance
1-Erythromycin	1	7	20	71.5%
2-Cefatriaxone	14	12	2	7.1%
3-Chloramphenicol	26	1	1	3.5%
4-Sulphamethoxazole/Trimethoprim	23	1	4	14.2%
5-Ciprofloxacin	27	0	1	3.5%
6- Azithromycin	25	1	2	7.1%
7- Amikacin	28	0	0	0
8- Streptomycin	15	0	13	46.4%

Antimicrobial susceptibility test: (Disk diffusion method), according to CLSI standards (2011):

All 28 Identified serotypes were tested for antibiotic resistance (selected 8 antibiotics)

Table 5. Frequency percentages and antibiotic resistance of identified *E. coli* Serotypes (n = 28)

SOURCE	Serotype	N^{O}	%	Resistant
Cheese	O44:K74	3	10.7%	E, S
	O26:K60	3	10.7%	E
	O145:K-	1	3.6%	-
	O115:K-	1	3.6%	E
	O8:K-	1	3.6%	S
Milk	O26:K60	3	10.7%	E, S
	O124:K72	2	7.14%	E
Minced meat	O145:K-	1	3.6%	S
	O158:K-	1	3.6%	-
	O86:K61	2	7.14%	E, S
	O124:K72	2	7.14%	E, S
	O25:K11	3	10.7%	E,C,STX,CIP,S
Children stool	O114:K90	3	10.7%	E
	O124:K86	2	7.14%	E,CRO,STX,AZM
Total		28	100%	

(E): Erythromycin; (CRO) Cefatriaxone; (C) Chloramphenicol; (CIP) Ciprofloxacin; (STX) Trimethoprim-Sulphamethoxazole; (AZM) Azithromycin (AK) Amikacin and (S) Streptomycin

Genotypic detection of *E. coli*:

Multiplex Polymerase Chain Reaction:

All 115 *E. coli* isolates were subjected for detection of Stx_1, Stx_2, eaeA and hlyA genes in a single reaction

Table 6. Incidence of virulence genes of isolated *E. coli* (n = 115)

		eae		Stx_1		Stx_2		hlyA	
Sample	No.	No	%	No	%	No	%	No	%
Meat	35	3	8.5	-	-	-	-	-	-
Milk	21	2	9.5	1	4.7	-	-	-	-
Cheese	31	1	3.2	-	-	1	3.20	-	-
Stool	28	2	7.1	-	-	1	3.60	-	-
Total	115	8	7.0	1	0.87	2	1.74	-	-

Table 3 results of antimicrobial susceptibility.

Multiplex PCR

The multiplex PCR detected targeted genes in 10 out of 115 (8.7%) collected samples. Seven samples possessed *eaeA* gene alone (70%); while 2 samples contained Stx_2 gene. Both *Stx1* and *eaeA* genes were detected in one sample.

Table 4 to 6 the illustrated results of multiplex PCR.

Discussion

E. coli, which are normal flora of the human and animal intestine, have been identified as a leading cause of food borne illness all over the world. *E. coli* strains have previously been isolated from meat samples and have also been implicated in the contamination of vegetables such as lettuce (Hilborn *et al*., 1999; Enabulele and Uraih, 2009). In our study, the incidence of *E. coli* was 35% in minced meat and 26% in raw milk and kareesh cheese. This was lower than that reported by other authors (Al-Gallasa *et al*., 2002; Hussein and Sakuma, 2005; Temelli *et al*., 2012); while it was higher than that reported by (Abdul-Raouf *et al*., 1996; Heuvelink *et al*., 1998; 1999; Dontorou *et al*., 2003). There are many factors affecting the differences in prevalence rates among studies; these differences are mainly related to samples and sampling (type, source/location and initial bacterial load), environmental and seasonal factors and the detection methodology used (Temelli *et al*., 2012). Usually high incidence is attributing to behavior of slaughtering and manipulation of food before reaching the market. Additionally, in this study *E. coli* incidence in diarrheal samples was 24.35% which almost the same reported in other studies (Nguyen *et al*., 2005; Bueris *et al*., 2007) and lower than that reported by (Keskimäki *et al*., 2000; Amisano *et al*., 2011). Regarding to Serotyping, the most identified isolates in our study were EHEC strains. Although *E. coli* O157:H7 was the most common EHEC strain in many regions of the world; serotypes O5, O26, O91, O103, O111, O113 and O145 were also recognized as a serious threat to public health and have been recovered from infected patients (Fagan *et al*., 1999; Perelle *et al*., 2007). Of the typeable *E. coli* isolates found, O26:K60 was the most common type with a percentage of 21.4 (6 isolates). Among the identified serotypes, O124:K72 was representing 14.3% which was higher than that reported in Kalyobia governorate, Egypt (Hassanien, 2004). In addition O44:K74, O114:K90 and O25:K11 are also found in 10.7%. In our study, no *E. coli* O157:H7 was isolated and that similar to that reported in other studies (Dontorou *et al*., 2003; Fantelli and Stephan, 2001).

Concerning the antibiotic susceptibility of the sero-identified strains. All strains were resistant to one or more tested antibiotics. This was similar to that conducted by (Radu *et al*., 2001). Twenty two out of 28 (78.57%) were resistant to at least one antibiotic and 6 strains (21.43%) were resistant to 3 or more antibiotics. These results show higher resistance profiles than that reported by (Dontorou *et al*., 2003). No antimicrobial resistance was observed against Amikacin (Aminoglycoside), while other studies showed resistance pattern that reach up to 10% (Rigobelo *et al*., 2010; Aly *et al*., 2012). Meanwhile resistance towards Erythromycin was 96.43%; which higher than that reported in other studies (Harakeha *et al*., 2005). This high percentage of Erythromycin resistance refers to its frequent use in our country. Such resistance may not only be a direct concern to human health, but also, it is important because it could be transferred to other important pathogenic serotypes (Dzidic and Bedeković, 2003).

By using the multiplex PCR for the detection of Stx_1, Stx_2, *eaeA* and EHEC *hlyA* virulence genes out of 115 isolates which were identifies as *E. coli* by biochemical tests, 10 samples showed positive results for the target genes. In the present study, eaeA positive *E. coli* isolates that were negative for other virulence genes were found to be predominant (70%). This result was higher than that reported in other studies (Dhanashree and Mallya, 2008; Sharifi-Yazdi, 2011). EHEC hlyA gene was not found in any of examined isolates; which contrary to what found in other studies (Paton and Paton, 1998; Dastmalchi and Ayremlou, 2012). Two isolates (20%) carried Stx_2 gene which lower than that reported by Sharifi-Yazdi *et al*. (2011; Dastmalchi and Ayremlou, 2012). One isolate only (10%) showed association between stx_1 and eaeA genes.

Conclusion

This study revealed that minced meat, raw milk and kareesh cheese were contaminated by *E. coli* and represented a public health hazard, therefore measures

should be taken to avoid such contamination and the product should be heat treated during manufacturing or before consuming. There is an increasing demand for improved diagnostic procedures for the detection of Shiga Toxigenic *Escherichia Coli* (STEC) in fecal samples and, in foods of animal origin because their ability to cause life-threatening diseases. However, considering the difficulty of performing phenotypic assay in some laboratories, the multiplex PCR can be used as a replacement of such conventional methods in detection of *E. coli* strains particularly the emerging ones.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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