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Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt

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ABSTRACT

The objectives of this study were to investigate prevalence and pathogenic potential of *Escherichia coli* contaminating raw milk and its products in Egypt. Out of 187 dairy products including 72 raw milk samples, 55 Karish cheese and 60 Ras cheese, 222 *E. coli* isolates including 111, 89 and 22 were obtained from 55 raw milk samples (76.4%), 41 Karish cheese (74.5%), and 13 Ras cheese (21.7%), respectively. Isolated *E. coli* strains were examined for 24 representative virulence genes present in diarrheagenic *E. coli* (DEC) and extraintestinal pathogenic *E. coli* (ExPEC). Among DEC and ExPEC virulence factors, genes for enteropathogenic *E. coli* (*eaeA*, *bfpA*, EAF), enterohemorrhagic *E. coli* (*stx1*, *stx2*, *eaeA*), enterotoxigenic *E. coli* (*elt*, *est*), enteroinvasive *E. coli* (*invE*), enteroaggregative *E. coli* (*Eagg*, *astA*), diffusely adherent *E. coli* (*daaD*), ExPEC (*cdt-I* to *cdt-V*, *cnf1*, *cnf2*, *hlyA*) and putative adhesins (*efa1*, *iha*, *ehaA*, *saa*, and $lpfA_{O113}$) were screened by colony hybridization assay. Out of 222 *E. coli* strains, 104 (46.8%) isolated from 69 (36.9%) samples carried one or more virulence genes. The most prevalent gene detected was $lpfA_{O113}$ (40.5%), followed by *ehaA* (32.4%), *astA* (3.15%), *iha* (1.80%), *hlyA* (1.35%), *stx1* (0.90%), *stx2* (0.90%), *eaeA* (0.45%), *cdt-III* (0.45%) and *cnf2* (0.45%). Two strains isolated from Karish cheese harbored 5 virulence genes (*stx1*, *stx2*, *iha*, *ehaA*, $lpfA_{O113}$). Stx subtype was determined to be *stx1* (not *stx1c* or *stx1d*) and *stx2d*. Indeed, expression of hemolysin A, CDT-III, CNF-II, Stx1 and Stx2d was confirmed by blood agar plate, cytotoxicity assay and Western blotting, respectively. Among the 222 *E. coli* strains, 54 (48.6%), 38 (42.6%) and 12 (54.7%) isolated from raw milk, Karish cheese and Ras cheese were potentially virulent, respectively. O-genotyping indicated that most of the potentially virulent *E. coli* isolates did not belong to clinically important O serogroups except O75, O91 and O166, which have been associated with human diseases. Phylogenetic grouping revealed that 150 (67.6%), 67 (30.2%) and 5 (2.30%) strains were clustered into A, B1 and D groups, respectively, which are considered to be associated with intestinal infection, indicating that these *E. coli* strains might have a potential to cause gastroenteritis. To the best of our knowledge, this is the first comprehensive study regarding prevalence and pathogenic potential of *E. coli* in dairy products in Egypt. Raw milk, Karish cheese and Ras cheese in Egypt are highly contaminated with *E. coli* including potentially pathogenic strains, which may impose a public health threat.

1. Introduction

Raw milk and cheese made from raw milk can be a major source of potentially harmful bacteria to human, such as pathogenic *Escherichia coli* (Oliver et al., 2005). Public health hazards associated with consumption of raw milk and raw-milk products, and related foodborne disease outbreaks have been reported in the world (De Buyser et al., 2001; Oliver et al., 2005). Although raw milk and raw-milk products have caused many illnesses and even deaths (FDA, 2012), their marketing and consumption widely exist in many countries including Egypt (Ayad et al., 2004; El Deeb et al., 2012). In Egypt raw milk is consumed especially in some rural areas and used to prepare cheese, known as Karish and Ras cheese. Karish is a fresh acid-coagulated soft cheese, and accounts for around 50% of white soft cheese produced in Egypt. Ras cheese is the most popular hard cheese in Egypt and it is manufactured in a high proportion under artisan conditions.

It is possible that milk and dairy products can be contaminated with a variety of microorganisms from different sources (Oliver et al., 2005). *E. coli* is one of these microorganisms, which is a normal inhabitant of large intestine in human and warm-blooded animals. The main source of *E. coli* in raw milk and milk products is fecal contamination during

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milking process along with poor hygienic practices. Therefore, *E. coli* is generally used as a reliable indicator of direct or indirect fecal contamination and the possible presence of enteric pathogens in raw milk and raw dairy products (Kornaki and Johnson, 2001).

However, some *E. coli* have acquired virulence genes rendering them pathogenic and can cause a variety of diseases both in animals and humans (Kaper et al., 2004). *E. coli* associated with human diseases can be broadly divided into two categories, intestinal and extra-intestinal infections, based on their distinct virulent properties and their clinical symptoms. *E. coli* causing intestinal infection is generally called diarrheagenic *E. coli* (DEC), which can further be subdivided into at least six categories, such as, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) based on their distinct pathogenic mechanisms and presence of pathotype-specific virulence genes (Kaper et al., 2004). In addition, it has been proposed that cytolethal distending toxin (CDT)-producing *E. coli* (CTEC) and *astA* gene-positive *E. coli* might be associated with diarrhea in human (Hinenoya et al., 2009; Yamasaki et al., 2006; Zhou et al., 2002).

On the other hand, extra-intestinal pathogenesis caused by *E. coli* (ExPEC) strains can be grouped into more or less three categories, namely, uropathogenic *E. coli* (UPEC) causing urinary tract infection (UTI), meningitis-associated *E. coli* (MNEC) and necrotoxigenic *E. coli* (NTEC) which produces cytotoxic necrotizing factor (CNF) (Kaper et al., 2004). These pathogenic *E. coli* strains have been isolated from diseased as well as healthy animals and humans (Belanger et al., 2011; Croxen et al., 2013; Kahali et al., 2004).

For many bacterial pathogens that infect mucosal tissues like gastrointestinal tracts, specific adhesins have been implicated as virulence factors. In *E. coli*, several genes encoding different adhesins such as *eaeA*, *efa1*, *iha*, *lpf*, and *ehaA* have been reported, which could be either pathotype-specific or irrespective of pathotype (Bardiau et al., 2010). Sometimes only one adhesin is expressed, which is critical for pathogenesis. Abolishment of the ability of a bacterial pathogen to attach and to colonize a specific tissue by adhesin-mediated receptor recognition is often enough to make it avirulent (Klemm and Schembri, 2000).

E. coli strains belonging to the same pathotype carry particular virulence determinants involved in the diseases. However, hybrid type of DEC, for example, with the characteristic of EHEC and EAEC has also been reported such as the German outbreak strain *E. coli* O104:H4 (Bielaszewska et al., 2011) and O111:H21 (Dallman et al., 2012). These strains, besides production of Stx, also possess virulence genes for EAEC. Therefore, monitoring of virulence gene profile of *E. coli* is important to detect newly emerging pathogenic *E. coli*. Apart from adhesins, several other virulence factors are also involved in *E. coli* pathogenesis including host cell surface-modifying factors, invasins, toxins and secretion systems, which are ideal targets for determination of pathogenic potentials of any given *E. coli* isolate (Kaper et al., 2004).

The possibility of transmission of pathogenic *E. coli* to humans and causing diseases, through consumption of raw milk as well as raw milk products, has been repeatedly documented worldwide (De Buyser et al., 2001). In fact, it has been reported that raw milk and raw milk products are contaminated with pathogenic *E. coli* not only in developing but also developed countries (Altalhi and Hassan, 2009; Jayarao and Henning, 2001; Paneto et al., 2007). However, prevalence of potentially pathogenic *E. coli* in raw milk and cheese made from raw milk in Egypt has not yet been examined. Bearing in mind the importance of *E. coli* as food-borne pathogens, and the important role of dairy products, especially cheese, as vehicle of human disease in the Egyptian diet, this study was designed to examine the occurrence of *E. coli* in commercial raw milk and cheese made from raw milk in Egypt and to investigate the prevalence of virulence genes in the *E. coli* isolates.

2. Material and methods

2.1. Sample collection

At the consumer level, 187 samples including 72 raw milk, 55 Karish cheese and 60 Ras cheese were randomly collected from local markets, farmer vendors and supermarkets at different localities in Nile Delta region: Menofia and El Beheira Governorates, Egypt, from April to November 2012. Sampling was focused in these governorates because many cheese producers are located in this region. Cheese types investigated in this study were mainly produced at farmers' houses (Karish cheese) and small factories (Ras cheese) from raw milk, where no starter culture is used for manufacturing these varieties of cheese. Two hundred and fifty grams of cheese sold without packaging were collected into sterile sample collection bag, transferred to the laboratory in a cool box with ice packs and analyzed immediately or kept at 4 °C for future analysis.

2.2. Culture and screening for E. coli

Ten milliliters from each raw milk and twenty five grams of each cheese sample were homogenized in a Stomacher-Blender (Seward Medical, London, England) with 90 and 225 ml of tryptic soy broth (TSB), respectively, and incubated at 37 °C for 16 h with shaking. Then, the cultures were streaked on eosin methylene blue (EMB) agar plates and incubated at 37 °C for 18–24 h for bacterial isolation. Three to five presumptive colonies (blue-black with a metallic green sheen) from each EMB plate were picked, streaked on tryptic soy agar (TSA) and incubated at 37 °C for 24 h. Then, the colonies were sub-cultured on nutrient agar slants at 37 °C for 16 h and used for future analysis. Species identification was done by colony morphology on EMB agar and using the following biochemical tests.

2.3. Biochemical tests

Biochemical identification of *E. coli* was done by using lysine indole motility medium (LIM), Simmons citrate agar, methyl red-Voges Proskauer medium (MR-VP), sulfide indole motility (SIM) medium and triple sugar iron agar (TSI) media. The tests were performed essentially as described elsewhere (Ewing, 1986).

2.4. Determination of O antigens

Somatic (O) antigens were determined by *E coli* O-genotyping PCR (Iguchi et al., 2015).

2.5. Detection of virulence genes by colony hybridization

Major virulence genes reported in pathogenic *E. coli* were screened by colony hybridization assay as described previously (Yamasaki et al., 1996) under high stringent condition (50% formamide in hybridization buffer and 65 °C incubation for washing) using ^{32}P-labeled DNA probes. DNA probes for *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *eaeA* (intimin), *bfpA* (bundle forming pilus), EAF (EPEC adherence factor), *est* (heat-stable enterotoxin), *elt* (heat-labile enterotoxin), *invE* (invasin of EIEC), *astA* (EAEC heat-stable enterotoxin 1), *Eagg* (aggregative adherence), *daaD* (fimbriae adhesin), *iha* (irgA homologue adhesion), *saa* (STEC autoagglutinating adhesion), *efa1* (*E. coli* factor for adherence), $lpfA_{O113}$ (long polar fimbriae), *ehaA* (EHEC autotransporter), *hlyA* (hemolysin), *cdtI-V* (cytolethal distending toxins I-V), *cnf1* (cytotoxic necrotizing factor 1) and *cnf2* (cytotoxic necrotizing factor 2) were prepared as described previously (Hinenoya et al., 2009). For *cdt* gene-probe, a mixture of PCR products of *cdt-IB*, *cdt-IIIB* and *cdt-IVB* genes, which could react with all the *cdt* types (I to V), were used (Hinenoya et al., 2009) because *cdt-III* genes are highly homologous to both *cdt-II* and *cdt-V*.