Critical occurrence of verotoxigenic *E.coli* and non-typhoidal *salmonella* in some heat treated dairy products

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Abstract

Pathogenic strains of *E.coli* and *Salmonella* are common causes of foodborne illness and have been frequently isolated from inadequately heat-treated milk products in Mansoura city. The current study was performed to explore the prevalence of *E.coli* and *Salmonella spp.* in heat-treated milk products intended for consumption in Mansoura university hospitals and hostels, as well as, to investigate their serotypes and virulence potential. Seventy-five samples of heat-treated milk products (Soft cheese, yoghurt, and processed cheese, 25 of each) were randomly gathered and directed to further investigation using conventional and molecular microbiology. Result revealed that 3(12%) of soft cheese samples harbored *E.coli* O146:H21, O26:H11 and O128:H2 serotypes and 2(8%) of yoghurt samples were contaminated with O128:H2 and O121:H7 serotypes while 3(12%) of processed cheese samples were positive for non-typhoidal *Salmonella* (NTS) serovars (*Salmonella* Typhimurium, *Salmonella* Infantis and *Salmonella* Essen). Virulence gene profiling reported that all *E.coli* isolates harbored *eaeA* gene and only *E.coli* O26:H11 and O121:H7 encoded *stx2* (verotoxin) gene. Further, all *Salmonella* isolates harbored *invA* and *stn* genes, while only *Salmonella* Typhimurium and *Salmonella* Infantis encoded *spvC* gene. This study confirmed the existence of highly pathogenic verotoxogenic *E.coli* (VTEC) and NTS in investigated milk products which could be hazardous for public health and resident in Mansoura hospitals and hostels. Hence, the implementation of good hygienic practices together with hazard analysis, and risk-based preventive control measures are rigorously required in the process of HACCP plan to eliminate the risk of contamination that may occur during the manufacturing process.

Introduction

Milk products are rich sources of necessary nutrients that achieve nutritional daily requirements either for the development and intense growth of children or for lessening the occurrence of chronic illnesses (osteoporosis, type II diabetes, hypertension, and cancer) in adolescents (Salles *et al*., 2019). lately, cheese constituted the main part of patient meals being an energy-rich nutritious, and a concentrated form of milk with the benefit of prolonged shelf life (El-Zamkan *et al.,* 2019). Moreover, yoghurt is a staple food in several cultures being an excellent source of probiotic microorganisms (viable *Lactobacillus acidophilus*) and lysozyme which can improve the immune response, antitumor effect, and encourage better assimilation of nutrients (Lourens-Hattingh and Viljoen, 2001). Despite this, milk products may harbor hazardous microbes such as *E. coli* and *Salmonella,* if they were improperly heat-treated and unsanitary processed which make them unfit or even threatening sources of foodborne illness (Singhal *et al*., 2020).

In fact, pathogenic strains of *E.coli* and *Salmonella* were identified frequently to be the common foodborne pathogens linked to consumption of raw or inadequately heat-treated milk products sold in rural areas and local groceries of Mansoura city (El-Baz *et al*., 2017; Omar *et al*., 2018; Elafify *et al*., 2020). The most prevalent *E.coli* seropathotypes related to human foodborne illnesses are; enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC) as well as enterohemorrhagic *E.coli* (EHEC) which is known as lethal shiga toxin or verotoxin producing *E.coli* (STEC or VTEC) (Hussien *et al*., 2019). STEC is a zoonotic diarrheagenic pathotype of *E.coli* causing approximately 2,801,000 moderate to severe gastrointestinal disorders, hemolytic uremic syndrome (HUS), and hemorrhagic colitis (HC) each year giving rise to serious public health burden worldwide (Galarce *et al*., 2019). The distinguishing characteristic of STEC is the presence of one or two major forms of Shiga-like toxins encoding genes such as *stx1* and *stx2* (verotoxin) genes together with enterohemolysin (*hly*) and intimin (*eaeA*) virulent genes that intensify its pathogenicity in causing infections (El-Baz *et al*., 2017). Although the majority of sporadic cases and outbreaks of STEC/EHEC are still attributed to O157:H7 serotype, nowadays the big six serotypes (O45, O26, O103, O121, O111, and O145) of non-O157 STEC showed a marked increase in incidence rates in Egypt (Elmonir *et al*., 2021) and worldwide (Gould *et al*., 2013; Loconsole *et al*., 2020).

By the same token, salmonellosis which is usually caused by *Salmonella* enterica, is an important zoonotic disease and the second most frequent foodborne illness which is incriminated in about 130 million cases of diarrheal outbreaks and about 450 deaths each year (Majowicz *et al*., 2010; Scallan *et al*., 2011). More than 2500 different serovars of *Salmonella* enterica have been identified, among them, *Salmonella* Typhimurium (*S.* Typhimurium), *Salmonella* Enteritidis (S. Enteritidis), and *Salmonella* Infantis (*S.* Infantis) were reported to be the most prevalent serovars in animal-based products in Egypt (Ahmed and Shimamoto, 2014) and across the world (Ferrari *et al*., 2019) representing a major public health concern due to their frequent worldwide isolation from humans with foodborne gastroenteritis (Herikstad *et al*., 2002). During the last few years, *S.* Infantis emerged as the fourth most prevalent cause of nontyphoidal salmonellosis (NTS) after *S.* Enteritidis, *S.* Typhimurium, and *S.* Hadar in Europe (EFSA and ECDC, 2018)

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and worldwide (Herikstad *et al*., 2002). *Salmonella* enteropathogenicity mainly related to their virulence genes like the chromosomally located invasion (*invA*) gene that facilitate adhesion and invasion of intestinal epithelial cells, the plasmid-encoded the fimbrial related gene (*spvC*) that exaggerate systemic spreading after inactivation and apoptosis of macrophage, and the chromosomally encoded enterotoxin (*stn*) gene that contributes in inducing severe diarrhea (Huehn *et al*., 2010; Omar *et al*., 2018; Saini *et al*., 2019). The purpose of this study was to investigate the safety state of heat-treated milk products commonly offered to the residents in Mansoura hospitals and hostels, and this by investigating the presence of *E. coli* and *Salmonella* strains in randomly collected milk product samples with attention to the characterization of their serotypes and virulence potential.

Materials and methods

Samples collection

A total number of seventy-five samples from different batches of dairy foods (Soft cheese, yoghurt, and processed cheese, 25 of each), were randomly collected in clean, dry, and sterile containers, from the food department of Mansoura university hospitals and hostels on the same day of receiving from the company of manufacturing, Dakahlia Governorates, Egypt at the period from June to October 2019. As reported in the label prescription, yoghurt and soft cheese samples were processed from pasteurized milk (not less than 63-68°C for 30 min) while processed cheese samples were cooked at a temperature higher than that used for pasteurization (75-85°C for 8-15 min) and this to minimize the risk of foodborne illness and provide acceptable shelf life. The obtained samples were immediately transferred in an ice tank at 4°C to our laboratory for microbiological investigation within few hours of the collection as specified by the ISO 6887-5:2010 method (ISO, 2010).

Microbiological screening of *E. coli* and *Salmonella*

Isolation of E. coli

In conformity with De Boer and Heuvelink (2000), each sample was ten-fold diluted (10^{-1}) using tryptone soya broth (Oxoid) and then incubated at 37°C for one day. From each diluted sample, one loopful was evenly speckled onto the surface of Eosin Methylene Blue (EMB) agar (Oxoid). After one day of aerobic incubation, 3 to 5 suspected *E. coli* colonies were picked and subcultured again on EMB agar and then incubated for another 24 hours at 37°C, then pure colonies were harvested and preserved at 4°C for further identification.

Isolation of Salmonella

Based on the method explained by Elafify *et al*. (2019), each sample was ten-fold diluted (10^{-1}) using buffered peptone water and then incubated at 37°C for one day. Then, 1ml of each diluted sample was transferred to 10 ml of Rappaport and Vassilidis enrichment broth followed by overnight incubation at 42°C. From each enrichment broth, one loopful was evenly streaked onto the surface of the xylose lysine desoxycholate (XLD) agar plate. After one day of aerobic incubation at 37°C, 3 to 5 suspected *Salmonella* colonies were picked and subcultured again on XLD agar and then incubated for another 24 hours at 37°C, then harvested pure colonies were preserved at 4°C for further identification.

Identification and characterization of the isolated *E.coli* and *Salmonella* strains

Biochemical identification

After refreshment of preserved isolates, biochemical identification was performed according to Hendriksen (2011) using triple sugar iron, indol production, hydrogen sulphide, citrate utilization, voges-proskauer, urease, and methyl red tests.

Serotyping

Biochemically confirmed *E.coli* and *Salmonella* strains were serologically typed into serovars by slide agglutination method using rapid diagnostic polyvalent and monovalent *E.coli* and *Salmonella* agglutinating antisera sets (Denka Seiken®, Tokyo, Japan) as per Forbes *et al*. (2007).

Molecular characterization

Bacterial genomic DNA extraction and purification were performed using QIAamp DNA Mini Kits (Cat#51304, Qiagen®, GmbH, Hilden, Germany) as per the manufacturer's protocol. In brief, each isolate suspended in enrichment broth (200 µl) was lysed for 2 h at 56°C with Qiagen® protease (20 µl) then lyses process was stopped by adding AL buffer (200µl) for 10 min at 56˚C. After centrifugation, 200 µl of ethanol (96%) was added to the DNA containing supernatant and the obtained mixture was passed through the QIAamp kit column. The purity of DNA bounded to a QIAamp membrane was improved after two washes using AW1 and AW2 (wash buffers). The purified DNA was then eluted in AE (50 µL) elution buffer and stored at -80°C until investigated for the presence of *E. coli* and *Salmonella* virulence genes.

PCR was applied via a thermocycler (Applied Biosystems Geneamp 2720) using 6 µl of the eluted DNA, 12.5 µl of Emerald Amp GT PCR master mix (2× premix,

Table 1. Oligonucleotide primers sequences used for virulence genes identification.

Identified bacteria	Target gene	Primer sequence (5'-3')	Products size (bp)	Reference
E.coli	*stx1* (F)	5'ACACTGGATGATCTCAGTGG3'	614	Elbaz *et al.*, (2019)
	stx1 (R)	5'CTGAATCCCCCTCCATTATG3'		
	stx2 (F)	5'CCATGACAACGGACAGCAGTT3'	779	
	stx2 (R)	5'CCTGTCAACTGAGCAGCACTTTG3'		
	eaeA (F)	5'ATGCTTAGTGCTGGTTTAGG3'	248	Bisi-Johnson *et al*., (2011)
	eaeA (R)	5'GCCTTCATCATTTCGCTTTC3'		
	hly (F)	5'AACAAGGATAAGCACTGTTCTGGCT3'	1177	Piva *et al*., (2003)
	hly (R)	5'ACCATATAAGCGGTCATTCCCGTCA3'		
Salmonella	*stn* (F)	5' GCTGTATTGTTGAGCGTCTGG 3'	617	Elafify *et al*., (2019)
	stn (R)	5' AGAAGAGCTTCGTTGAATGTCC3'		
	invA (F)	5'GTGAAATTATCGCCACGTTCGGGCAA3'	284	Bisi-Johnson *et al.,* (2011)
	invA (R)	5'TCATCGCACCGTCAAAGGAACC3'		
	spvC (F)	5'ACCAGAGACATTGCCTTCC3'	467	Huehn *et al.,* (2010)
	spvC (R)	5'TTCTGATCGCCGCTATTCG3'		

stx1, shiga-toxin 1 gene; *stx2,* shiga-toxin 2 gene; *eaeA*, intimin gene; *hly*, hemolysin gene; *invA*, invasion protein gene; *stn*, *Salmonella* enterotoxin gene; *spvC*, *Salmonella* plasmid virulence gene; F, forward primer; R, reverse primer; bp, base pair.

TAKARA Bio Inc. Cat# RR310A), 1 µl of each forward and reverse primer as well as 4.5 µl of PCR grade water. The primer pairs (Table 1) that were used for the identification of virulence genes were designated by Metabion (Martinsried, Germany) whereas; positive control strains were obtained from Animal Health Research Institute, Dokki, Egypt.

The amplification reaction was applied as per the following protocol: primary denaturation at 94 °C for 5 min; followed by 35 cycles of secondary denaturation at 94 °C for 30 sec; annealing at 58 °C for 40 sec for *stx1*, *stx2* and *spvC* at 51 °C for 30 sec for *eaeA*, at 60 °C for 40 sec for *hly*, at 59 °C for 40 sec for *stn* and 55 °C for 30 sec for *invA* gene; extension at 72 °C for 45 s in *stx1*, *stx2*, *stn,* and *spvC*, at 72 °C for 30 s in *eaeA* and *invA*, at 72 °C for 60 s in *hly*. Finally, the PCR amplified products were visualized under UV light and photographed after electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) stained with ethidium bromide.

Results and discussion

The lack of sanitary measures and individual hygiene during the manufacturing of some dairy products makes them the primary reservoir of *E.coli* that represents an insidious threat to human health and food safety (Momtaz *et al*., 2012). The result of our investigation (Table 2) revealed that 3/25 (12%) of examined soft cheese samples were positive for *E.coli* and this incidence nearly agrees with Stephan *et al*. (2008), and Elbaz *et al*. (2019) who revealed that 5/52 (9.6%) and 3/30 (10%) of examined samples in Switzerland and Egypt, respectively, were positive for *E.coli*. Another study from Brazil stated the high prevalence of *E.coli* (95.5%) especially O26:H11 serotype in different soft cheese brands. In our study, *E.coli* isolates were serologically identified as O146:H21, O26:H11 and O128:H2 serovars that similar to serotypes previously detected in soft cheese during another survey in the same city by Elhadidy and Mohammed (2013) who isolated O146:H21 and O26:H11 whereas, O128:H2 was previously isolated from soft cheese in Iran (Momtaz *et al*., 2012). In the same way, our study showed that 2/25 (8%) of examined yoghurt samples were tainted with *E.coli* O128:H2 and O121:H7 serotypes that also identified in other study conducted in Iran by Dehkordi *et al*. (2014).

Hence, based on our survey and the most recent result of *E. coli* serotypes prevalence in yoghurt (Elbaz *et al*., 2019) and soft cheese (Elafify *et al*., 2020; Elbaz *et al*., 2019) sold at the retail level in Mansoura city, we noted that it is the second time to isolate O146 and O26 from soft cheese since seven years ago by Elhadidy and Mohammed (2013) and it is the first time to isolate O128 and O121 from yoghurt in the same city.

In an attempt to assess the pathogenicity of these serotypes, virulence factors were further determined by PCR and our findings (Table 2, Figure 1) confirmed the presence of *eaeA* virulence gene in all isolated serotypes that have a role in enhancing immediate adherence of microorganism to the intestinal wall resulting in the attaching and effacing (A/E) lesions after disruption of intestinal microvillus brush border (Blanco *et al*., 2005). Despite this, our result stated the low pathogenicity of EPEC (O128 and O146) due to the absence of *stx2,* s*tx1*and *hly* virulence genes but also, our results confirmed the emergence of highly pathogenic non-O157 VTEC in soft cheese (O26) and yoghurt (O121) as they harbored and expressed *stx2* (Verotoxin) virulence gene that usually implicated as a cause of sporadic cases or outbreaks of bloody diarrhea, HUS and renal insufficiency as it thousand times more toxic for renal microvascular endothelial cells than *stx1* especially in the presence of *eaeA* gene that has an accessory role in augmenting the pathogenicity of STEC (EFSA, 2013).

Henceforth, virulence gene profile suggested that STEC positive products were manufactured from improperly pasteurized milk that previously polluted either from the farm environment or contaminant in the dairy chain as Cattle are a major reservoir of highly virulent STEC strains that carry *stx2* and *eaeA* with high rates than *stx1* and *hly* (Karama *et al*., 2019).

Non-typhoidal *Salmonella* is a major microbiological hazard associated with the consumption of dairy products made either

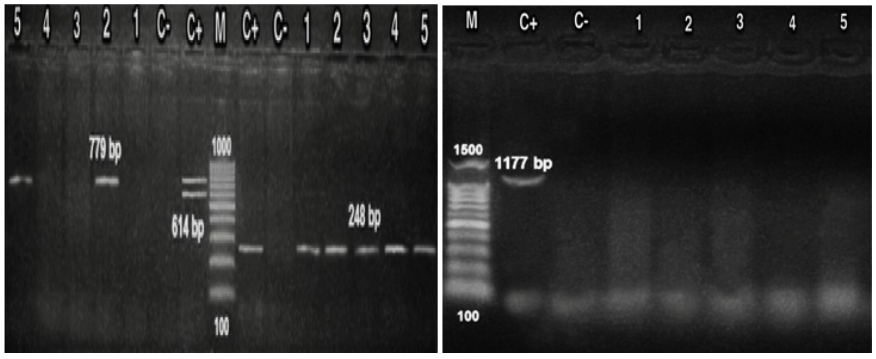

Figure 1. Agarose gel electrophoresis of representative PCR products for amplification of *stx1* (614 bp), *stx2* (779 bp), *eaeA* (248 bp), and *hly* (1177 bp) virulence genes of isolated *E. coli*. Lane M: 100 bp ladder as molecular size DNA marker; lane C+: Positive control *E. coli* for *stx1*, *stx2*, *eaeA*, and *hly* genes; lane C-: Negative control (without DNA); lanes1(O146) and 3, 4 (O128): positive *E. coli eaeA* gene; lanes 2(O26) and 5(O121): Positive *E. coli* for *eaeA* and *Stx2* genes; Lanes1(O146), 2(O26), 3(O128), 4(O128) and 5(O121): negative *E. coli* for *hly* gene.

Table 2. Prevalence, Serodiagnosis, and Virulence genes of *E.coli* isolated from soft cheese and yoghurt that consumed in Mansoura university hostels and hospitals.

Type of samples (No.)	Prevalence No. (%)	Pathotype	Serodiagnosis	Virulence genes			
				stx1	*stx2*	*eaeA*	*hly*
Soft cheese (25)	3 (12)	EPEC	O146:H21	-	-	+	-
		STEC	O26:H11	-	+	+	-
		EPEC	O128:H2	-	-	+	-
Yoghurt (25)	2 (8)	EPEC	O128:H2	-	-	+	-
		STEC	O121:H7	-	+	+	-

stx1, shiga-toxin 1 gene; *stx2*, shiga-toxin 2 gene; *eaeA*, intimin gene; *hly,* hemolysin gene.

Table 3. Prevalence, Serodiagnosis, and Virulence genes of *Salmonella* spp. isolated from processed cheese that consumed in Mansoura university hostels and hospitals.

Type of samples (No.)	Prevalence No. (%)	Pathotype	Group	Somatic (O) antigen	Flagellar (H) antigen Phase 1	Phase 2	Virulence genes *spvC*	*stn*	*invA*
Processed cheese (25)	3(12)	*S. Typhimurium*	B	1,4,5,12	1	1,2	+	+	+
		S. Infantis	C1	6,7	R	1,5	+	+	+
		S. Essen	B	4,12	g,m	-	-	+	+

invA, invasion protein gene; *stn, Salmonella* enterotoxin gene; *spvC, Salmonella* plasmid virulence gene.

from raw milk or milk subjected to post-pasteurization contamination by food handlers mainly in developing countries with poor hygienic standards (WHO, 2015). Despite the negative result of *salmonella* species in all studies concerned with the microbiological examination of processed cheese worldwide (Kim *et al*., 2018), the culture method in our study revealed (Table 3) the presence of *Salmonella* in 3/25 (12%) of processed cheese samples that were serologically identified as *S.* Typhimurium, *S.* Infantis and *S.* Essen which globally reported as the most frequent cause of human salmonellosis and foodborne gastroenteritis outbreaks (Hendriksen *et al*., 2011). The presence of *salmonella* species in our result might be owned to the fact that processed cheeses encounter several conventional materials which have high potent to carry microbial contaminant of animal or human fecal wastes especially if it processed in unhygienic conditions at a lower temperature than that required for pasteurization (Kim *et al*., 2018).

It is worth noting that other investigated dairy products in Mansoura city were in harmony with our finding, where *S.* Typhimurium and *S.* Infantis were observed in 4% of soft cheese and Kareish cheese by El-Baz *et al*. (2017) and recently three isolates of *S.* Typhimurium were isolated from Kareish cheese by Elafify *et al*. (2019). To author knowledge, this is the first study reporting the isolation of very rare *S.* Essen serotype from the milk-based product as it commonly isolated only from retail food and chicken meat (Menghistu *et al*., 2011; Wang *et al.,* 2015) and was of public health importance for both human and veterinary medicine.

Of note, the pathogenicity potential of *Salmonella* spp. depends mainly on the presence of the genetic determinants responsible for their virulence (Rhen *et al*., 2007; Saini *et al*., 2019). In the current study, PCR based assay proves the ability of isolated *S.* Typhimurium and *S.* Infantis to invade, destroy, escape from macrophage and colonize host intestinal cell inducing various lesions and gastrointestinal signs if consumed within infected dairy products as

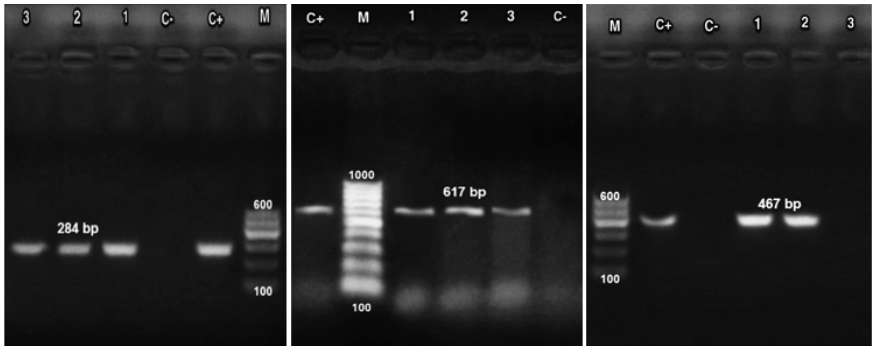

Figure 2. Agarose gel electrophoresis of representative PCR products for amplification of *invA* (284 bp), *Stn* (617 bp), and *spvC* (467 bp) virulence genes of isolated *Salmonella*. Lane M: 100 bp ladder as molecular size DNA marker; lane C+: Positive control *Salmonella* for *invA*, *stn*, and *spvC* genes; lane C-: Negative control (without DNA); lanes1 (*S. Typhimurium*), 2 (*S. Infantis*), and 3 (*S. Essen*): positive *Salmonella* for *invA* and *stn* genes; lanes1 (*S. Typhimurium)* and 2 (*S. Infantis*): Positive *Salmonella* for *spvC* genes; lanes 3 (*S. Essen*): negative *Salmonella* for *spvC* genes.

they were positive for *invA, stn* and *spvC* virulence genes (Table 3, Figure 2) which were also identified previously by El-Baz *et al*. (2017), Omar *et al*. (2018) and Elafify *et al*. (2019) in the same serotypes but in other conventional milk products. Astonishingly, PCR screening in our work confirmed that *S.* Essen serotype poses infection risks to humans as despite being negative for *spvC* virulence gene which responsible for systemic infection, it was positive for *invA* and *stn* virulence genes which are mandatory for *Salmonella* enterotoxigenic potency in inducing humans gastroenteritis.

Conclusions

Conclusively, This study declares the existence of highly pathogenic non-O157 VTEC and *Salmonella* spp. in dairy products every day supplied to the resident in Mansoura hospitals and hostels which constitute a potential risk to the public health. Hence, the implementation of good hygiene together with hazard analysis, and risk-based preventive control measures are rigorously required in the process of HACCP plan to eliminate the risk of contamination during processing rather than reliance on end-product testing.

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