Molecular characterization of *Escherichia coli* isolated from cheese and biocontrol of Shiga toxigenic *E. coli* with essential oils

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Abstract

The current research was carried out to study the incidence of *Escherichia co*li (*E. coli*) in Egyptian cheese (Kariesh and Ras) and molecular characterization of certain *E. coli* virulence genes (*stx1, stx2, eaeA, hlyA and fimH*) using multiplex PCR technique. Biocontrol of *E. coli* with essential oils (clove and thyme oil) was also studied. A total of 150 random samples of Kariesh and Ras cheese (75 each) were collected from various areas in Governorate of Menoufia. According to our results, the frequency of *E. coli* isolated from Kariesh and Ras cheese was 16% and 5.3%, respectively. Serological identification classified the *E. coli* strains into two groups, enterohemorrhagic *E. coli* (EHEC) serogroup (O26: H11, O91: H21, O111: H2 and O103: H2). While the enterotoxigenic *E. coli* (ETEC) serogroup were detected as O125: H21 which is the most prevalent strain. O171: H2, O86 and O119: H6 belonging to enteropathogenic *E. coli* (EPEC). The most prevalent gene detected in *E. coli* strains was *stx1* (87.5%) followed by *stx2* (86%), *fimH* (75%), *hlyA* (50%) and *eaeA* (25%) genes. Concerning the antimicrobial activity with essential oils, thyme oil (1%) is considered as the bactericidal effect against *E. coli* (ATCC35150) with improved the sensory evaluation than clove oil (1%). In conclusion, Kariesh and Ras cheese are extremely tainted with pathogenic *E. coli* strains, which represent a strong hazard on the human health.

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Introduction

Food safety is considered one of the most common urgent matters in the food industry worldwide. Spoilage of the food products with foodborne pathogens receive a special concern among food producers, investigators and customers. Consequently, producing a safe food represents one of the most imperative urgencies in the food processing (Friedman *et al*., 2002; Mohamed *et al*., 2013). The outbreaks of foodborne illnesses caused by *E. coli* have been studied previously in developing countries after ingestion of milk products such as old-style cheese which is considered the major source of various types of pathogenic bacteria (Elhadidy and Mohammed, 2013). *E. coli* is one of the major significant bacteria, which has a bad effect on both human and animal species. Thus, this type of bacteria can deteriorate the milk particularly raw milk and other milk products as a result of poor hygienic measures (Lara *et al*., 2016; Garbaj *et al*., 2016).

E. coli is classified into six pathotypes: enteroaggregative, enterohemorrhagic/ Shiga toxin-producing *E. coli* (STEC), enteroinvasive, enteropathogenic, enterotoxigenic, and diffuse adherent (Jafari *et al*., 2012). lethal STEC named EHEC were also detected (Beutin *et al*., 2007). Previous studies indicated that STEC represents one of the most significant pathotypes which lead to foodborne illnesses compared with other types *E. coli* (Brett *et al*., 2003; Kaufmann *et al*., 2006). In human, the pathogenic effect of STEC nearly due to its ability to produce certain types of cytotoxins for example Shiga toxins (*stx1* and *stx2*), enterohemolysin (*hly*) and intimin (*eae*) virulent genes (Slanec *et al*., 2009 and Assumpção *et al*., 2015).

Among the dairy products, cheese is considered one of the most public sources of vital nutrients (*e.g.* vitamins, minerals and proteins) which represent the main part of healthy food (López-Expósito *et al.,* 2012). In Egypt, Ras cheese is a hard cheese prepared from milk of large animals. It needs a prolonged time, 90-95% humidity and about 12°C to be prepared (El-Hofi *et al*., 2010). Moreover, Kariesh cheese is another prevalent type of cheese which contains a higher protein content with a small amount of fats (Hamad, 2015). Nevertheless, cheese is considered a safe food for human being, sometimes its deterioration by various types of foodborne pathogens may take place. *Listeria monocytogenes*, *Salmonella* and enteropathogenic *E. coli* (EPEC) are considered the most common bacteria isolated from cheese. EHEC such as *E. coli* O157:H7 may also cause high morbidity and mortality rates among young and old people (Kousta *et al*., 2010).

Essential oils (EO) are known to have antibacterial and antioxidant effects (Yousefi *et al*., 2017). Numerous researches reported that EO have a potent antibacterial effect against different types of pathogens which indicated their ability to protect the foodstuffs (Burt, 2004; Kotzekidou *et al*., 2008; Yahyazadeh *et al*., 2008; Lee *et al*., 2010; Bajpai *et al*., 2012 and Jeong *et al*., 2014). Various EO with multiple effects such as antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and carcinopreventive effects have been established

previously by Chorianopoulos *et al*. (2008) and São Pedro *et al*. (2013). From the previously revealed data, the current research was achieved to isolate and identify the pathogenic *E. coli* and recognition of its virulence genes (*e.g. stx1, stx2, hlyA eaeA and fimH*) using multiplex PCR technique as well as studying the ability and effectiveness of essential oils extracted from thyme, clove and laurel plant on isolated pathogenic *E. coli* in soft cheese.

Materials and Methods

Sample collection

One hundred fifty samples including Egyptian Kariesh cheese (n=75) and Ras cheese (n=75) were collected randomly from various markets in certain areas in Menoufia Governorate and preserved in the ice box for culturing process within two hours of collection.

Isolation of *E. coli*

According to the method described by De Boer and Heuvelink (2000), the isolation of *E. coli* was carried out. In brief, 25 grams of each specimen were moved to sterile tube comprising 225 mL of tryptiase soya broth (TSB, Oxoid, UK) and then stored at 37°C for 18-24 hours. From the broth of each incubated tube, one loopful was speckled onto the Eosin Methylene Blue agar (EMB, Oxoid, England). After two days of incubation, the probable colonies were sub-cultured and then incubated for another two days at 37°C for further identification.

Serological identification of *E. coli*

The identified *E. coli* isolates were serologically typed using slide agglutination test (standard polyvalent and monovalent *E. coli* antisera) based on the method described by Edwards and Ewing (1972).

Molecular characterization of STEC strains

DNA Extraction

QIAamp kits were used for extraction of DNA from *E. coli* isolates, according to the technique described previously by Hessain *et al.* (2015).

Primer sequences used for identification of E. coli virulence genes

The Shiga toxins (*stx1* & *stx2*), intimin (*eaeA*), hemolysin (*hlyA*) and D-Mannose-specific adhesion "type 1 fimbriae" (*fimH*) virulent genes of *E. coli* were amplified according to the technique recommended by Fagan *et al*. (1999) using designated primers (Pharmacia Biotech) as shown in Table 1. The amplification of *fimH* gene was carried out according to Pusz *et al*. (2014)

In vitro susceptibility testing of thyme and clove essential oils on isolated strains of *E. coli* and ATCC35150

Preparation of bacterial strains

Stock cultures of 16 *E. coli* strains that obtained from our present study were preserved in nutrient Broth (NB, Oxoid, UK) at 4°C. Microorganism inoculum was fortified in NB at 37°C for 24 h. Peptone Water (Oxoid CM0009) was used to dilute the cell suspension to provide 10^6 CFU/mL (Celikel and Kavas, 2008). ATTCC 35150 (E. coli O157:H7) strain EDL931 genome (GenBank accession no. AWXM00000000.2) was also used in our study.

Extraction of essential oil

From dry clove and thyme plants, the extraction of active ingredients were achieved based on the method described by (Tandon and Rane, 2008).

Preparation of Kariesh cheese

According to El-Khawas and Hassan (2015), cow's milk was got from the College of Veterinary Medicine, Benha University, Egypt in which the percentage of fat's milk is 4.2% (AOAC, 2000). Milk was pasteurized at 75ºC for 15 seconds, there after cooled to 43ºC then inoculated with 3% (v/v) of yoghurt starter culture. All treatments were incubated at 37ºC, up to curding. The combination was divided into four 8 pars as: (I); Control (no essential oils or bacterial strains are present), salt at 1% was added between cheese layers and the curd was left to whey drain into small cheese molds at 22-25°C and the mixture was then divided into four 4 parts as follow: (I); Control without antimicrobials or biological agent; (II) *E. coli* strain with CFU at 10^6/mL; (III) *E. coli* with thyme oil 0.5%; (IV) *E. coli* with clove; (V) *E. coli* with thyme oil 1%; (VI) *E. coli* with clove oil 1%. Two parts of clove 1% and thyme oils were set for sensory assessment (1% for each oil), Cheeses from various handlings were kept in firmly locked plastic bottles and enclosed with whey at 6±2°C for two weeks.

Cheese samples examination

The cheese specimens were tested after two weeks for sensory evaluation and *E. coli* counting. All tests were accomplished in three replicates and the mean values were then measured. Sensory evaluation for both control and treated Kariesh cheese specimens was performed based on the method suggested by Clark *et al.* (2009). The samples were assessed for flavor, body and texture, color and appearance. Ten grams of cheese sample was transferred to 90 mL of diluents containing 2% of sodium citrate (Sigma-Aldrich, USA) for preparation the cheese homogenate. One mL of main dilution was then moved to 10 mL of diluents to get sequential dilutions (ISO, standard DIS 6887-5, 2010). One mL of the serial dilutions was moved onto two plates of Eosin

Table 1. Primer sequences used for identification of *E. coli* virulence genes.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F) *stx1* (R)	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Dhanashree and Mallya (2008)
stx2 (F) *stx2* (R)	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Dhanashree and Mallya (2008)
eaeA (F) *eaeA* (R)	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	890	Mazaheri *et al.* (2014)
hylA (F) *hylA* (R)	ACGATGTGGTTTATTCTGGA CTTCACGTGACCATACATAT	165	Fratamico *et al.*. (1995)
fimH (F) *fimH* (R)	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	165	Chapman *et al.* (2006)

Methylene Blue Agar (Oxoid, UK) for bacterial counting. Subsequently, the plates were kept at 37°C for 24-48 hours. Distinctive *E. coli* colonies were calculated and recorded according to APHA (2004). The analysis of variance (ANOVA) test was carried out to investigate the statistical significance ($P \leq 0.05$).

Results and Discussion

Dairy products are liable to be contaminated from different sources during production, contamination and their presence in food lead to be unfit for consumption and constitute a public health hazard (Virpari *et al*., 2013). In our research, the incidence of *E. coli* was 16% and 5.33% in examined Kariesh and Ras cheese samples, respectively from (75 examined samples of each). For Kariesh cheese, higher result (74.5%) was obtained by Farhad *et al*. (2017). For Ras cheese, higher results (28%) was obtained by Virpari *et al*. (2013) and Farhad et al. (2017). Lower result (11.54%) for kareish cheese was recorded by Farhad *et al*. (2017).

The main factors which affect on the quality and composition may be as a result of the clotted skimmed milk, the process of production, the period needed to complete the whey drain, the superiority of the added salt and the practice of management complete cheese (Aldo *et al*., 2013). The incidence of *E. coli* in in our samples may be as a result of deficiency of appropriate hygiene and lack of sterilization of milk utilized for cheese manufacture.

In our article we identified the EPEC, ETEC and EHEC serogroups in samples of cheese and the EHEC was considered one of the major predominant serogroup (Table 2). In this study EHEC serogroup were detected as O26: H11, O91: H21, O111: H2 and O103: H2. While ETEC serogroup was detected as O125: H21 which is the most prevalent strain. O171: H2, O86 and O119: H6 belonging to EPEC. Ladan and Reza (2006) indicated that O119 represents one of the dominant EPEC serogroup recovered from cheese.

E. coli bacteria contain multiple virulence genes that encourage its establishment and attack of the human cells (Ejrnæs, 2011). There are other virulence genes in *E. coli* strains such as toxins which is a secretary virulence factors and the most important of these factors is α hemolysin, this factor encoded by hly gene (Bien *et al*., 2012). The Multiplex PCR was used for the recognition of *stx1, stx2, eaeA* and *hlyA* virulence genes in 16 *E. coli* isolates. As shown in

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

Product	Serodiagnosis	Strain characterization
Kareish cheese	3 O125 : H21	ETEC
	O171 : H2	EPEC
	O86	EPEC
	3 O26 : H11	EHEC
	O91 : H21	EHEC
	O111 : H2	EHEC
	O156 : H7	EPEC
	O103 : H2	EHEC
Ras cheese	O119 : H6	EPEC
	O111 : H2	EHEC
	O26 : H11	EHEC
	O91 : H21	EHEC

Table 3. Incidence of virulence genes of EPEC strains isolated from the examined samples.

No. examined isolates	*stx1*		*stx2*		*eaeA*		*hlyA*		*fimH*	
	No.	%	No.	%	No.	%	No.	%	No.	%
16	14	87.5	11	68	4	25	8	50	12	75

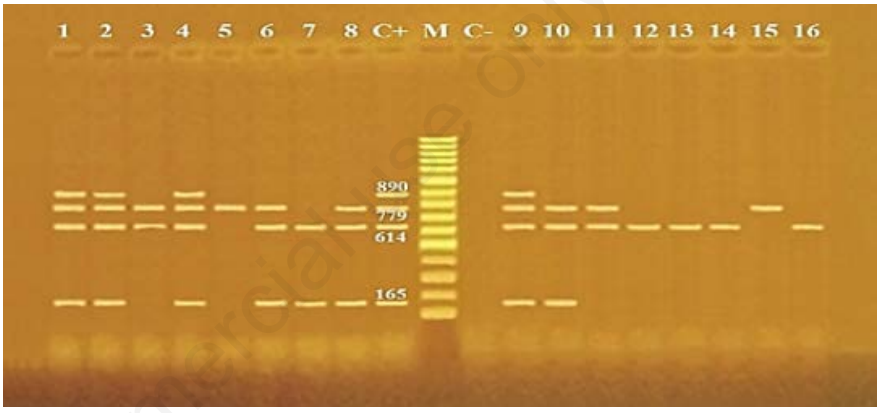

Figure 1. Agarose gel electrophoresis of multiplex PCR of *stx1* (614 bp), *stx2* (779 bp), *eaeA* (890 bp) and *hlyA* (165 bp). Lane M: 100 bp ladder as molecular size DNA marker; lane C+: Control positive E. coli for stx1, *stx2, eaeA* and *hlyA* genes; lane C-: negative control; lanes 1, 2, 4 (O26) and 9 (O111): positive *E. coli* for *stx1, stx2, eaeA* and *hlyA* genes; lanes 6 (O91), 8 (O103) and 10 (O111): positive *E. coli* for *stx1, stx2* and *hlyA* genes; lanes 3 (O26) and 11 (O119): positive *E. coli* for *stx1* and *stx2* genes; lanes 7 (O91): positive *E. coli* for *stx1* and *hlyA* genes; lanes 12, 13, 14 (O125) and 16 (O171): positive *E. coli* for *stx1* gene; lanes 5 (O86) and 15 (O156): positive *E. coli* for *stx2* gene.

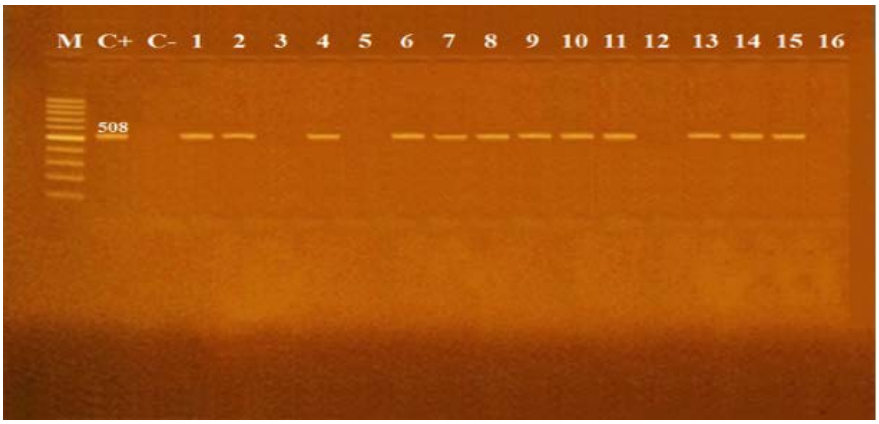

Figure 2. Agarose gel electrophoresis of PCR of *fimH* (508 bp). Lane M: 100 bp ladder as molecular size DNA marker; lane C+: control positive *E. coli* for *fimH* gene; lane C-: control negative; lanes 1, 2, 4 (O26); 6, 7 (O91); 8 (O103); 9, 10 (O111); 11 (O119); 13, 14 (O125) and 15 (O156): positive *E. coli* for *fimH* gene. Lanes 3(O26); 5 (O86); 12 (O125) & 16 (O127): negative *E. coli* for *fimH* gene.

Table 3 and Figure 1, 14 (87.5%), 11 (86%), 4 (25%), 8 (50%) of isolates contains *stx1, stx2, eaeA* and *hlyA* virulence genes, respectively. Whereas; 12 (75%) of isolates contain *fimH* (Figure 2). Similar results regarding the prevalence of *stx1* producing strains were described previously by Martin and Beutin (2011). Lower incidence of *stx1, stx2* was recorded by Virpari *et al*. (2013). 15 % and 22.50% of *E. coli* isolates found positive for *stx1* and *stx2* genes, respectively. Higher incidence was obtained by Elhadidy and Mohammed (2013) who reported that all recovered isolates contain 100% *stx1* and *stx2* genes whereas *eae* gene was excited in 21% of *E. coli* isolates, which is nearly similar to our study (25%). In contrast, Farhad *et al*. (2017) couldn't detect any isolate has *eae* gene. The *eae* gene is necessary for the attachment of bacterium with epithelial cells (Vu-Khac *et al*., 2006). The pathogenic effect of STEC is related to the production of *stx1* and *stx2* genes as verocytotoxins. Elhadidy and Mohammed (2013) stated that *stx2* gene is considered one of the furthermost important virulence genes and the majority of hemolytic-uremic disorder in individuals are caused mainly by STEC strains that carry *stx2* gene. The *eaeA* gene is an additional virulence gene for STEC that was important to increase the pathogenicity of STEC. However, the *eaeA* gene hasn't been detected in some STEC strains that cause human diseases (Kruger and Lucchesi, 2015). Furthermore, Douellou *et al*. (2017) showed that the virulence gene profiles of dairy products were similar to human STEC strains.

Biocontrol of *E. coli* strains (O26) by thyme and clove essential oils was also investigated in our study. The statistics demonstrated in Tables 4 and 5 indicated that the counts of *E. coli* (O26) were gradually decreased from zero time $3.0\times10^6 \pm 0.2\times10^6$, $2.2\times10^5 \pm 0.1\times10^5$ and $5.7\times10^4 \pm 1.0\times10^4$ in cheese sample with thyme oil 0.5% with reduction % reach to 92.7% at 1st week and 98.1% while in cheese sample with 1% thyme oil the reduction % reach 99.8% at 1st week and disappear at 2nd week of refrigerated storage. Similar results were described by Al Maqtari *et al*. (2011) who stated that the *Staphylococcus aureus* and *E. coli* strains were highly susceptible to the thyme oil and exhibited an imperative antimicrobial effect.

The reduction percentages were in cheese sample with 0.5% clove oil 73.7% and 98.1at 1st and 2nd week with mean value $7.9\times10^5 \pm 1.2\times10^5$ and $2.8\times10^5 \pm 0.3\times10^5$, respectively, while in samples with 1% clove oil the reduction % were 92.3 and 98.5 in 1st and 2nd week with mean value $2.3\times10^5 \pm 0.1\times10^5$ and $4.5\times10^4 \pm 0.8\times10^4$, respectively. While in control group (cheese with *E. coli* only) the count of *E. coli* still high from zero time to 2nd week of refrigerated storage with mean $3.0\times10^6 \pm 0.2\times10^6$, $2.6\times10^6 \pm 0.1\times10^6$ and $2.4\times10^6 \pm 0.1\times10^6$, respectively. These results agree with that reported by Ayah and Saad (2016). According to our results, the antimicrobial activity with essential oils, thyme oil (1%) is considered as the bactericidal effect against *E. coli* (ATCC35150) with improved the sensory evaluation than clove oil (1%). The usage of higher concentration of *in vivo* EOs than *in vitro* may be as a result of the more complex growth environment in foodstuffs, which play an important role in the microbial cells protection from antimi-

Table 4. The effect of different essential oils (0.5%) on *E. coli* (O26) count (CFU/g) inoculated into Kariesh cheese.

Storage time	Strain only		Thyme oil		Clove oil	
	Count	R %*	Count	R %	Count	R %
Zero time	$3.0\times10^6 \pm 0.2\times10^6$	-	$3.0\times10^6 \pm 0.2\times10^6$	-	$3.0\times10^6 \pm 0.2\times10^6$	-
1st week	$2.6\times10^6 \pm 0.1\times10^6$	13.30	$2.2\times10^5 \pm 0.1\times10^5$	92.70	$7.9\times10^5 \pm 1.2\times10^5$	73.70
2nd weeks	$2.4\times10^6 \pm 0.1\times10^6$	20.00	$5.7\times10^4 \pm 1.0\times10^4$	98.10	$2.8\times10^5 \pm 0.3\times10^5$	90.60

R %*= Reduction %

Table 5. The effect of different essential oils (1%) on *E. coli* count (CFU/g) inoculated into kariesh cheese.

Storage time	Strain only		Thyme oil		Clove oil	
	Count	R %*	Count	R %	Count	R %
Zero time	$3.0\times10^6 \pm 0.2\times10^6$	-	$3.0\times10^6 \pm 0.2\times10^6$	-	$3.0\times10^6 \pm 0.2\times10^6$	-
1 week	$2.6\times10^6 \pm 0.1\times10^6$	13.3	$3.6\times10^3 \pm 0.5\times10^3$	99.8	$2.3\times10^5 \pm 0.1\times10^5$	92.3
2 weeks	$2.3\times10^6 \pm 0.1\times10^6$	20.0	ND	-	$4.5\times10^4 \pm 0.8\times10^4$	98.5

R %*= Reduction %

Table 6. Sensory evaluation scores of fresh manufactured soft cheese treated with essential oils (1%).

Group	Traits			
	Flavor (30)	Texture (60)	Appearance and color (10)	Over all (100)
Fresh cheese (zero time)				
Control	22	54	9	85
Clove oil	24	53	9	86
Thyme oil	26	55	9	90
1st week				
Control	22	52	8	83
Clove oil	26	53	9	88
Thyme oil	28	55	9	92
2nd week				
Control	S	S	S	S
Clove oil	25	50	8	83
Thyme oil	26	54	8	88

S: Spoiled samples.

crobial agents (Marija and Nevena, 2009). The bactericidal effect of EOs may due to their ability of cellular wall degradation, cell membrane damage, obliteration of membrane proteins and enhanced permeability of the cell membrane leading to escape the different ions and other contents of the bacterial cell (Nazzaro *et al*., 2013). Thyme oil is recognized to has antibacterial effect against various microorganisms including *E. coli* isolated from foodstuffs. Smith-Palmer *et al*. (2001) and Burt and Reinders (2003) indicated that thyme oil has bacteriostatic and bactericidal effects against *E. coli* O157: H7.

The first impression about food is usually visible, and the most important thing regarding the consumer's willingness to consume the food is based mainly on its inspection. Frequently if its appearance is unappealing, the consumer doesn't accept any other characteristics such as flavor and texture (Gambaro *et al*., 2001). The scores for sensory assessment of fresh kariesh cheese hand-made by different methods are listed in Table 6. At first two weeks of storage, a high flavor score was detected in the thyme oil cheese specimens, whereas a reduced value was detected in clove oil cheese specimens at the 2nd week of refrigerated storage. After adding EOs, no momentous influence on the texture value. The entire value of cheese specimens was significantly ($P<0.05$) increased at zero day with both thyme and clove oils. A greater sensory value was detected in cheese with thyme oil in the first two weeks of storage compared with the control and clove oil usage, whereas the lowermost score was stated in control samples at the 1st week. Similar results were obtained by Ismail *et al*. (2006). White cheese treated with essential oils had softer consistency than in the control group; because the existence of EOs in cheese can improve the enzymatic action (Mervat *et al*., 2010).

Conclusions

From the above-mentioned results, it can be clarified the public health importance of pathogenic *E. coli* and its virulence genes that were determined in our study in milk products (Kariesh and Ras cheese) in Egypt, that might be attributed to contamination which might be explained by improper sanitation, lack of health education and lack awareness about efficient control measures. Furthermore, contamination of milk and milk products as a foodborne zoonosis are remained a constant public concern with various implications in Egypt.

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