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Detection of Shiga Toxin – Producing *Escherichia coli* in Raw and Pasteurized Milk

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Abstract

One hundred random samples of raw buffalo and pasteurized cow milk (50, each) were collected from different localities of Sharkia Governorate for the detection of *E. coli*. The isolates were screened by PCR for virulence associated genes as well as antibiotic sensitivity test to determine the most effective antimicrobial agent. Furthermore, an experimental study was carried out to detect the influence of pasteurization and processing of kariesh cheese and yoghurt on *E. coli* O_{111}:H_8 inoculated in milk with the concentration of 10^8 CFU/mL. The obtained results showed that the occurrence of *E. coli* in the examined raw buffalo and pasteurized cow milk samples were 66% and 30%, respectively. Serogrouping of *E. coli* isolates revealed that O128, O26 and O111 were recorded as the most frequent O-serogroups. The sensitivity test showed that *E. coli* isolates were more sensitive to gentamicin (79.2%), followed by ciprofloxacin (70.8%) and colistin (68.8%). However, the examined isolates were completely resistant to erythromycin (100%) followed by sulphamethazole- trimethoprim (79.2%). Molecular identification of virulence associated genes revealed *stx*1, *stx*2 and *eae*A genes. The experimental study showed that milk pasteurization was more effective on *E. coli* $\mathbf{O_{111}}$:$\mathbf{H_8}$ survival at refrigeration temperature compared with processed products such as kariesh cheese and yoghurt.

Keywords: *E. coli*, Serotypes, *stx*1, *stx*2, *eae*A

Introduction

In the recent years, there is an increasing demand for high quality natural food free from contaminating pathogens and artificial preservatives. Raw buffalo milk and its products such as cheese and yogurt are widely consumed in Egypt. Contamination of raw buffalo milk and milk products with pathogenic bacteria occurs mainly during unhygienic milking process, handling and transportation [1].

Escherichia coli is recognized as a foodborne pathogen colonizing the large intestine and produces different types of toxins including shiga like toxins (Stx) responsible for severe hemorrhagic colitis in humans [2]. Some members of shiga toxin-producing *Escherichia coli* (STEC) groups have been proved to be widely associated with both outbreaks and sporadic cases of foodborne diseases in humans, ranging from complicated diarrhea to a life-threatening complication known as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) [1].

Shigatoxigenic *E. coli* produce Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) that bind and enter eukaryotic cells via the receptor of glycolipid and inhibit protein synthesis, leading to cell death [3].

Shiga toxin-producing *E. coli* serotype O_{157}:H_7 is considered as one of the most important known foodborne microorganisms due to the severity of associated illnesses despite the reported low infective dose (less than 10 cells) [4]. In addition, non-O_{157} STEC strains belonging to other serogroups including O_{26}, O_{91}, O_{103}, O_{111}, O_{128} and O_{145} have been reported in milk and dairy products causing severe illness in humans [5-7].

Previous studies have shown that milk can be contaminated during unhygienic milking process [8]. Although milk pasteurization removes almost all pathogenic *E. coli*, inactivation of *E. coli* shiga toxins has not been proven. However, following consumption of pasteurized milk, an outbreak in North Cumbria, England, during 1999 with HUS

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cases have been reported and no living bacteria were found in the milk samples [9].

The aim of the present investigation is to detect shiga toxin producing *E coli* and their genes (*stx*1, *stx*2 and *eae*A) in raw buffalo milk and pasteurized cow milk. Determination of the most effective antibiotic on the recovered isolates was carried out. In addition, the effect of pasteurization and manufacture on the survival of *E. coli* O_{111}:H_8 at refrigeration temperature was studied.

Material and Methods

Collection and preparation of samples

Raw buffalo and pasteurized cow milk samples (50, each) were randomly collected from different localities in Sharkia Governorate, Egypt. The volume of each milk sample was 50 mL. All the collected samples were placed in an ice box and transported immediately as soon as possible to the laboratory of Microbiology at Animal Health Research Institute (Zagazig Branch) for bacteriological examination.

Bacteriological examination

Isolation of E. coli

Milk samples were centrifuged at 3000 rpm and 1 mL of the sediment was steaked onto MacConkey agar (Oxoid) and incubated aerobically at 37°C for 24 hours. Subculture was made onto MacConkey agar and Eosin Methylene Blue (EMB, Oxoid) for purification of the isolates. Hemolytic strains were confirmed by streaking the pure colonies on Blood agar [10]. Suspected colonies were then picked up for biochemical identification.

Biochemical identification

Purified colonies were biochemically identified using API20 BioMerieux, Marcy I'Etoile, France as previously described [10,11]. The used tests were the indole, methyl red, Voges-Proskauer and citrate tests (IMViC).

Serological identification

Serotyping of *E. coli* was done at the Faculty of Veterinary Medicine, Benha University, Egypt. The *E. coli* immune-O-sera (8 polyvalent sera vials and 43 vials hyper monovalent antisera) were obtained from DENKA SEIKEN Co. LTD, Tokyo, Japan. *E. coli* serotyping was carried out according to Edward and Ewing [12].

Antimicrobial susceptibility test

The susceptibility test for *E. coli* isolates to nine antibiotics was conducted using disc diffusion test according to National Committee for Clinical Laboratory Standards (NCCLS) [13]. The used agents provided from Oxoid were ciprofloxacin (5 µg), gentamicin (10 µg), colstin (10 µg), doxycycline (10 µg), erythromycin (15 µg), Sulphamethazole-Trimethoprim (10 µg), Amoxicillin-Clavulinic acid (20/10 µg), streptomycin (10µg) and chloramphenicol (30 µg). The inhibition zone was then measured and interpretation was carried out according to Clinical and Laboratory Standards Institute (CLSI, 2011) [14].

Molecular characterization of virulence associated genes

The investigated virulence associated genes (*stx*1, *stx*2 and *eae*A) were amplified using the specific primers stx1-F (5'-ACA CTG GAT GAT CTC AGT GG-3'); R (5'-CTG AAT CCC CCT CCA TTA TG-3' which produced 614 bp product [15], stx2-F (5'-CCA TGA CAA CGG ACA GCA GTT-3'); R (5'-CCT GTC AAC TGA GCA GCA CTT TG-3') that produced 779 bp [15] and eaeA-F (5'-GTG GCG AAT ACT GGC GAG ACT-3'), R (5'-TAA ATC CAC GCC CAG TCG CAA AAA-3') that produced 890 bp product [16].

The DNA was extracted from the suspected colonies using QIAamp DNA Mini Kit according to the manufacturer's guidelines (Qiagen, Germany, GmbH). A multiplex PCR using the three pairs of primers was used for the amplification as reported by Fagan *et al.* [17]. The reaction was carried out in a final volume of 50 µL containing 2 µL of DNA, 10mM Tris-HCl (pH 8.4), 10mM KCl, 3 mM MgCl2; 2 mM concentrations of each primer, 0.2 mM concentrations of each 29-deoxynucleotide 59-triphosphate, and 4 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification cycling conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 sec, 58°C for 40sec, and 72°C for 90 sec. The final cycle was followed by 72°C incubation for 5

min. The reference strains were *E. coli* O_{111}:H_8 Sakai (positive for *stx*1, *stx*2 and *eae*A*)* and *E. coli* K12DH5α (a nonpathogenic negative control strain) that does not possess any virulence gene, both were kindly obtained from Microbiology Department, Benha University. The amplified DNA fragments were then electrophoresed in 2% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment size.

Experimental study

An isolated and identified *E. coli* O_{111}:H_8 strain during the current study was used to investigate the impact of pasteurization and processing on the survival of the isolate. The bacterial inoculum was prepared on nutrient agar plates; colonies were separately transferred using a sterile loop to a tube containing 5mL of sterile saline solution. The turbidity of this tube was adjusted to match a McFarland obesity tube No. 0.5 ($1.5x10^8$CFU/mL) by adding sterile saline. The adjusted bacterial broth was inoculated into 600 mL of raw buffalo milk that was bacteriologically examined and was free from *E. coli*. The inoculated milk sample was then divided to 3 portions: the first portion was pasteurized [18], the second portion was used to process kariesh cheese [19] and the third portion was used to process yoghurt [20]. Control groups of non-inoculated milk and milk products were also included. Pasteurized milk and milk product samples were kept at refrigeration temperature (4-6°C) and bacteriological examination was conducted at zero day, 1st day and 2nd day to determine *E. coli* O_{111}:H_8 count using duplicate sampling from each product twice daily. From each sample, 10 mL were added to 90 mL of 0.1% peptone water homogenized. The homogenate was serially (10 folds) diluted in 0.1% peptone water. Volumes of 0.1mL of the diluted samples were surface plated in duplicate onto MacConkey agar (Oxoid). After incubation for 24 h at 37ºC, all sorbitol fermented colonies on plates were enumerated [21]. Bacterial count in each type of samples is given as mean values and standard deviations.

Statistical analysis

The difference between the pasteurization and processing at refrigeration storage for three periods of times was estimated using two-way ANOVA test (Factorial design) and LSD (Least significant difference). The test results were calculated by SPSS version 22 (IBM Corp. 2013, Armonk, NY). Data were presented as mean±SD and significance was considered at $P \leq 0.01$.

Table 1: Serological identification of *E. coli* isolates from raw buffalo and pasteurized milk

Source	O type (somatic)	H type (flagellar)
Pasteurized milk	O_{128}	H_2
Raw milk	O_{91}	H_{21}
Pasteurized milk	O^{128}	H_2
Raw milk	O_{44}	H_{18}
Raw milk	O_{26}	H_{11}
Raw milk	O_{26}	H_{11}
Raw milk	O_{111}	H_8

Results and Discussion

The obtained results revealed that the isolation rates of *E. coli* in the examined raw buffalo and pasteurized cow milk samples were 66% (n=33) and 30% (n=15), respectively. These results were in accordance with Ali and Warda [22] who detected *E. coli* in raw cow's milk samples with percentages varied from 45-80%. However**,** Yadav *et al.* [23] obtained lower isolation rate of *E. coli* in raw milk (33.3%) than those obtained in the current study. Whereas, *E. coli* was isolated from 40.8% and 50% of the local and brand pasteurized milk samples, respectively [24]. In addition, Soomro *et al.* [25] reported that the prevalence of *E. coli* in milk and dairy products was 51.7% and 57%, respectively.

Table 2: Sensitivity and resistant patterns of *E. coli* isolates from raw buffalo and pasteurized milk (n= 48)

Antibiotics	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Gentamycin	38	79.2	0.0	0.0	10	20.8
Ciprofloxacin	34	70.8	5	10.4	9	18.8
Colistin	33	68.8	5	10.4	10	20.8
Doxycycline	24	50.0	4	8.3	20	41.7
Erythromycin	0.0	0.0	0.0	0.0	48	100
Sulphamethazole-Trimethoprim	5	10.4	5	10.4	38	79.2
Amoxicillin- Clavulinic acid	24	50	4	8.3	20	41.7
Streptomycin	29	60.4	5	10.4	14	29.2
Chloromphenicol	20	41.7	5	10.4	23	47.9

Representative 13 *E. coli* isolates were sero-grouped as shown in Table (1). Four different serotypes were identified from raw buffalo milk samples as following: O_{26} (2 strains), O_{44} (1 strain), O91 (1 strain) and O_{111} (1 strain), in addition to four untypable *E. coli* isolates. While, one *E. coli* serotype from pasteurized cow milk was identified as O128 (2 isolates) and two isolates were untypable. This is in accordance with Perelle *et al.* [26] who mentioned that O_{128}, O_{26} and O_{111} are the most frequent O- serogroups involved in food poisoning outbreaks. However, other serotypes, such as *E. coli* O_{26}:H_{11}, O_{103}:H_2, O_{145}:H_{28}, and O_{111}:H_8, were implicated in outbreaks [27]. STEC strains of these O-serogroups are considered as the world's main pathogenic strains implicated for public health hazards.

The results of antimicrobial susceptibility test on *E. coli* isolates (n=48) showed that they were highly sensitive to gentamicin (79.2%) followed by ciprofloxacin (70.8%) and colistin (68.8%), respectively (Table 2). All the isolates were completely resistant to erythromycin (100%) followed by sulphamethazole -trimethoprim (79.2%) and chloramphenicol (47.9%). These results coincided with those obtained by Lin *et al.* [28] who recorded that *E. coli* isolates showed high rates of resistance to erythromycin (89.4%) and exhibited high sensitivity to gentamycin. In contrary, *E. coli* isolates from foods were highly sensitive to kanamycin (80%), followed by chloramphenicol (60%) and the least sensitivity was shown against ciprofloxacin (40%), nitrofurantoin (40%) and streptomycin (20%) [29].

Table 3: Distribution of *stx*1, *stx*2 and *eae*A genes in the examined *E. coli* isolates from raw buffalo and pasteurized milk determined by PCR

E. coli serogroup	*stx*1	*stx*2	*eae*A
O_{26}:H_{11}	+	+	+
O_{26}:H_{11}	+	+	-
O_{44}:H_{18}	-	+	-
O_{91}:H_{21}	+	+	-
$O_{11}1$:H_8	+	+	+
O_{128}:H_2	+	-	-
O_{128}:H_2	+	-	-

Molecular characterization of virulence associated genes was carried out on the typable seven isolates (Table 3). The results of molecular identification of *stx*1, *stx*2 and *eae*A virulence associated genes revealed that were 85.7%, 42.9% and 28.6% of the examined isolates harbored the three genes, respectively, and *stx*1 was the most detected one. This result agreed with those reported by Sheikh *et al.* [30] who detected *stx*1, *stx*2 *and eae*A genes in the examined samples of milk and milk products.

The obtained results in Table (4) showed that milk pasteurization and processing of kariesh cheese and yoghurt reduced the count of previously inoculated *E. coli* O_{111}:H_8 (10^8 CFU/ mL) in raw milk significantly. The statistical analysis exhibited that the mean *E. coli* count in the pasteurized milk significantly decreased comparing with those in the other two milk products. These results were explained by the relatively higher thermal effects against bacterial growth in the pasteurized milk comparing with those in yogurt and kariesh cheese processing. These results coincided with those reported by D'Aoust *et al.* [31] who reported significant reduction of *E. coli* count in milk after pasteurization.

Table 4: Counts (Mean ±SE) of inoculated *E. coli* O_{111}:H_8 in raw milk after pasteurization and kariesh cheese and yogurt processing

Storage duration (days)	Number of *E. coli* O_{111}:H_8 (CFU/mL or gm)		
	Pasteurized milk	**Kariesh cheese**	**Yoghurt**
0	$0.97X\ 10^2 \pm 0.66X\ 10^{d}$	$2.0X\ 10^5 \pm 4.2\ X10^{3a}$	$3.5\ 10^5 \pm 3.8\ X10^{4a}$
1	$1.0X\ 10^3 \pm 0.5\ X10^{2c}$	$1.0X\ 10^5 \pm 2.8\ X10^{3a}$	$2.0\ X10^3 \pm 0.67\ X10^{2c}$
2	$9.8X\ 10^3 \pm 2.8\ X10^{2b}$	$2.0X\ 10^5 \pm 8.3\ X10^{3a}$	$4.8X\ 10^5 \pm 3.5\ X10^{4a}$

Initial count inoculated in raw milk is 10^8 CFU/ mL. Means carrying different superscripts are significantly different at (P-value ≤ 0.01), while means carrying similar superscripts are insignificantly different based on Least Significant Difference (LSD).

The bacterial count was then increased significantly by the time from the zero day to the 1st and 2nd days in the pasteurized milk. Whereas, the bacterial count was significantly decreased in the yogurt at 1st day compared with those in zero and 2nd days. Concerning kariesh cheese samples, non-significant difference of *E. coli* counts within the different durations was observed. These findings could be attributed to the decreased pH levels during yogurt and kariesh cheese processing, and subsequently reduced the bacterial count. Likewise, *E. coli* disappeared in plain yoghurt after 5 days of storage in another study [32].

Conclusion

In conclusion, the pasteurized milk is relatively safer rather than raw milk regarding the occurrence of *E. coli.* Moreover, the milk pasteurization exhibited more effect against *E. coli* O_{111}:H_8 count comparing with their processing of kariesh cheese and yogurt.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

الكشف عن بكتيريا الميكروب القولوني المعوي(الايشرشيا القولونية) المنتجه لسموم الشيجا في الحليب الخام و اللبن المبستر

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تم جميع ١٠٠ عينة عشوائية من لبن الجاموس الخام والحليب البقرى المبستر (٥٠ لكل منهما) من مدن مختلفة من محافظة الشرقية للكشف عن ميكروب الايشرشيا القولونية وكذلك استخدام تقنية تفاعل انزيم البلمرة المتسلسل(PCR) لإستبيان وجود جينات الضراوة بالعزلات. فضلا على إجراء اختبارات الحساسية لتحديد المضادات البكتيرية الأكثر فعالية ضد المعزولات. وقد أجريت الدراسة التجريبية للكشف عن تأثير البسترة و صنيع الجبن القريش و الزبادي على تواجد بكتيريا الايشرشيا القولونية O_{111} H_8 المحقونه في الحليب بتركيز ١٠ ^{٨} خليه/ملي من البكتيريا المذكورة. وقد أظهرت النتائج تواجد بكتيريا الايشرشيا القولونية في ٣٣ عينه (٦٦%) من عينات الحليب الخام و ١٥ عينه فقط (٣٠%) من الحليب المبستر. وبعد التحليل النوعى لمجاميع الانتيجين (o) باستخدام اختبار التلزن لهذه المعزولات كانت مجاميع الانتيجين O_{128}، O_{26} وO_{111} من السلالات O- الأكثر شيوعا في العينات التي تم فحصها. واظهرت المعزولات حساسيه اكثر للجنتاميسين (٧٩.١%)، يليه سيبروفلوكساسين وكوليستين ٧٠.٨٪ و ٦٨.٨٪ على التوالي. وعلى الجانب الاخراظهرت المعزولات مقاومة عالية ماما للإريثروميسين (١٠٠%)، يليه سلفاميثازول مع راي ميثوبريم (٧٩.٢%) . كما أظهر فحص تفاعل انزيم البلمرة المتسلسل (PCR) للمعزولات تواجد جينات *stx*1، *stx*2 و*eae*A وقد كشفت الدراسة التجريبية أن بسترة الحليب أظهرت تأثير أكثر ضد تواجدالايشرشيا القولونية O_{111}:H_8 المحقونه في اللبن المستخدم قبل البستره مقارنة مع صنيع الجبن القريش و الزبادي المصنعين من نفس اللبن .