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Coliform Contamination of Marketable Milk Sold in New Valley Governorate, Egypt

Enas El-Prince[1], Asmaa M.H. Sayed[1], Rania M. Ewida[2*]

[1]Department of Food Hygiene (Milk Hygiene), Faculty of Veterinary Medicine, Assiut University, Egypt.

[2]Department of Food Hygiene, Safety and Technology (Milk Hygiene), Faculty of Veterinary Medicine, New Valley University, Egypt.

***Correspondence**
Rania M. Ewida
E-mail address: r_ewida@vet.nvu.edu.eg

Abstract

This study was performed to evaluate the hygienic condition of marketable milk sold in New Valley Governorate by determination of coliforms contamination. One hundred and fifty marketable milk samples were collected from farmer's houses, dairy farms and dairy shops and these samples were examined for coliform counts. The coliforms percentages were 20, 6 and 14% for concerning samples with average counts of $1.9X10^4$, $6.6X10^4$ and $4.6X10^4$cfu/ml, respectively. Moreover, *Escherichia coli* was found in 2, 2 and 4% of the analyzed milk samples, respectively. The serotypes of *E. coli* strains were O127:K63, O55, O114:K90 and O44:K74. Two strains carried intmin gene and all isolates didn't contain hemolysin gene. On the other side, *Enterobacter* spp. was detected in percentages of 4, 2 and 4% in the farmer's houses, dairy farms and dairy shop milk samples, respectively. Moreover, *Cronobacter sakazakii* was detected in 7(14%) of farmer's houses and 4(8%) of dairy shop milk samples and it couldn't be detected in dairy farms milk samples. The coliform contamination in marketable milk in New Valley Governorate reflects the unhygienic condition of milk production, therefore strict hygienic measures must be applied during milk production to safeguard consumers.

KEYWORDS
Marketable milk, *E. coli*, *Enterobacter* spp., *Cronobacter sakazakii*

INTRODUCTION

Milk is a perfect food (Abd El-Aal, 2000) because it contains protein, fat, vitamins, sugar and minerals. So, it is considered a very good medium for different microorganisms as coliforms (Ruegg, 2003).

Coliforms are one of the indicator organisms which declared the unsanitary conditions of milk practices such as the health of the udder milk, milkers, and utensils sanitation (Reinemann *et al*., 1997; Murphy and Boor, 2000; Davidson *et al*., 2004). Because it is simple and inexpensive to do, and it frequently correlates with the population of other bacteria in bulk tank milk, the coliform count is a useful indicator of milking cleanliness (Jayarao *et al*., 2004; Pantoja *et al*., 2009). Coliforms are Gram-negative, aerobic, and facultative anaerobes, non-spore forming rods, and can ferment lactose to lactic acid and gas at 35-37°C (Davidson *et al*., 2004).

Coliforms divide into three groups (1) psychrotolerat environment coliforms as Serratia and Hafnia this group can multiply in milk at refrigerator temperatures (Masiello *et al*., 2016) (2) thermotolerant fecal coliforms as *E. coli* which is normally inhabit at mammalian intestine, so *E. coli* is not considered as environmental contamination. (3) ubiquitous coliforms genera as *Enterobacter* and Citrobacter which may derive from two sources; fecal matter and environmental sources (Trmčić *et al*., 2016).

Coliform bacteria contain many genera which affect public health as well as it has a spoilage effect in dairy products (Chambers, 2002; Mandell *et al*., 2005) as *E. coli*. It is one of the most pathogens that can cause gastrointestinal or urinary tract infections in humans and animals, in addition, it is one of the most causative organisms that cause mastitis in dairy animals (Ali *et al*., 2017).

Another genus of coliforms is *Enterobacter* which has been recorded as one of the most serious opportunistic pathogens for humans also it was reported in several outbreaks of hospital-acquired infectious around the world which may be reached to become the majority of nosocomially acquired illnesses (Huang *et al*., 2001).

Cronobacter spp. Was first defined as *Enterobacter sakazakii* by Farmer *et al*. (1980) after that it was reclassified by Iversen *et al*. (2008) and Joseph *et al*. (2012a, b). It is a novel pathogen that affects children, particularly infants, as well as the elderly (Hunter and Bean, 2013). The main clinical signs that occur by *C. sakazakii* are meningitis or necrotizing enteritis in infants, Additionally, septicemia, diarrhea, and urinary tract infections have also been reported (Block *et al*., 2002; Bowen and Braden, 2006).

On the other side, psychrotolerant coliforms can produce several spoilage enzymes as lipolytic and proteolytic enzymes (Nornberg *et al*., 2009; Masiello *et al*., 2016) which are responsible for many faults in the finished milk products as flavor, odor, and body defects. Finally, milk containing coliforms results in poor quality milk and detrimental effect on consumer health.

Therefore, the current study was created to identify coliform contamination in marketable milk sold in New Valley Governorate, Egypt.

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MATERIALS AND METHODS

Ethical approval

The regulations of the ethics committee in the Faculty of Veterinary Medicine at New Valley University, Egypt were followed in terms of animal care and study protocol.

Samples and study area

In clean, dry sterile containers, 150 random samples of marketable milk including farmer's houses, dairy farms, and dairy shops (50 samples each) were collected from New Valley Governorate, Egypt during the period from January to February 2020. The collected samples (about 250 ml of milk) were transferred to the Milk Hygiene Department laboratory immediately as possible to be analyzed.

Preparation of samples

According to A.P.H.A. (2004), the samples were well-mixed and only one ml from each sample was used for the examination.

Determination of coliforms

Enrichment procedure (Samadpour *et al*., 1990)

Each prepared sample was inoculated aseptically into sterile test tubes with 10 ml of EC broth using 3 ml of the sample. The inoculated tubes were incubated at 37°C for 24 h.

Plating on selective agar media (De Boor and Heuvelink, 2000)

A loopful of the incubated broth was streaked onto MacConkey agar plates and incubated at 37°C for 24h. Colonies showing circular pink with spreading growth. Suspected colonies were transferred to nutrient agar slants and incubated at 37°C for 24h

Enumeration of coliforms (A.P.H.A. 2004)

1 ml of each dilution was transferred and inoculated in 15 ml from melted, sterilized, and tempered Violet Red Bile (VRB) Agar, and thorough*hly* mixed. After complete solidification of the medium, the plates were incubated at 37°C for 24-48h in the inverted position and the counts were presented as colony-forming unit per ml (cfu /ml). Suspected colonies were transferred to nutrient agar slants and incubated at 37°C for 24h.

Identification of coliforms

Biochemical tests (A.P.H.A., 2004)

As motility test, catalase test, triple sugar iron (TSI), methyl red test, indol test, Voges-Proskaur test and Citrate utilization test.

Serological tests (Edwards and Ewing, 1972)

Standard polyvalent and monovalent EC antisera were used in the serological laboratory of the Animal Health Research Institute (Cairo, Egypt) to serotype *E. coli* isolates using a slide agglutination test.

Polymerase chain reaction

DNA extraction

The strains were subcultured onto tryptic soya broth (TSB) and incubated overnight at 37°C for DNA extraction using Qiagen DNA blood Mini kit (Cat. No. 51104, Hilden, Germany) according to product instruction. At -20°C, the DNA that was extracted was kept.

DNA amplification

It was carried out in a final volume of 25 µl and the following ingredients were used: 12.5 µl of 2X PCR master mix (Green Master, Promega, USA), 150 ng of DNA template, 1 µl of each primer (Table 1), and water free of nuclease to bring the reaction to a final volume of 25 µl. The ingredients were mixed in a PCR tube. In a programmable gradient thermal cycler (Veriti Applied Biosystem, USA), The pathogenic genes of *E. coli* amplification were carried out at 94°C for five minutes, followed by 35 cycles that were completed under the following conditions: denaturation at 94°C for 50 sec, annealing at 63°C for 50 sec, and extension at 72 °C for one min. Finally, the mixture was subject for 10 min at 72°C as a final extension. While the PCR condition of 16S rRNA of *C. sakazakii* amplification was done as follows: two min of DNA denaturation, put up by 30 cycles were run under the next conditions; denaturation at 94°C for the 30s, annealing at 60°C for 1min and extension at 72°C for 90s. The final extension was done for 5 min at 72°C.

Gel Electrophoresis

PCR products were electrophoresed in 1% agarose gel (GX 040.90, Gen Agarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno–Train Diagnostic, D–61476, Kronberg/ Taunus) with ethidium bromide as 1µl /ml electrophoresis buffer at 100 volts for 60 min. The gel was inoculated with 100 bp DNA–ladder (SciE–PLAS, HU 10, 5636, UK), samples and controls. The result was carried out by high-performance ultraviolet Transilluminator, (UV, INC, UK). The photo of the target genes was analyzed by DOC–It® LS, Image acquisition–software, (Biodoc Analyzer, Biometra, Germany).

Table 1. Primers used to amplify fragments of genes responsible for expression of different virulence factors for *E. coli* and 16S rRNA gene of *C. sakazakii*.

Primer Name	Sequence (5'-3')	Target gene	PCR product (bp)	Reference
AE22	ATTACCATCCACACAGACGGT	*eaeA*	397	Sarimehmetoglu *et al*. (2009)
AE20-2	ACAGCGTGGTTGGATCAACCT			
MFS1-F	ACGATGGTTTATTCTGGA	*hly*	166	
MFS1-R	CTTCACGTCATCACCATACATAT			
Esak –F	GCT YTG CTG ACG AGTGGC GG	*16S rRNA*	929	Angelika and Roger (2004)
Esak -R	ATC TCT GCA GGA TTCTCT GG			

Statistical analysis

Data were entered into Microsoft Excel Spreadsheet.

RESULTS AND DISCUSSION

Coliform is a class of Gram-negative bacteria which ferment lactose of milk and produce lactic acid and gas. A sign of milk contamination is the presence of coliforms in it due to improper handling of either milk or milk utensils (El-Bakri and EL-Zubeir, 2009).

In the current work, the results illustrated in Table 2, revealed that 20, 6 and 14% of the collected milk samples were contaminated with coliforms from farmers' houses, dairy farms, and dairy shops, respectively. The high percentage of coliforms in farmer's houses' milk samples may be attributed to manual milking, poor hygienic practices during milking and storage, also the ineffective cleaning and sanitization of equipment.

The average counts of coliforms were $1.9x10^4$, $6.6x10^4$ and $4.0x10^4$ cfu/ml for the concerning examined milk samples, respectively. The highest count ranged from 10^4 to 10^5 cfu/ml for all examined milk samples.

As shown in Table 3, the obtained results are lower than that carried out by Arafa (2013) and Fathi *et al.* (2019). However, it is higher than the outcomes pointed out by El-Leboudy *et al.* (2014).

The illustrated findings in Table 4, showed that the prevalence of *E. coli* isolated from farmer's houses and dairy farms samples each was 1 (2%), while in dairy shops milk samples were 2 (4%). These results did not comply with that stated by Egyptian Standards (2010).

The first pathotype of *E. coli* is enteropathogenic *E. coli* (EPEC) which mainly causes acute diarrhea, vomiting and low-grade fever and affect child younger than two years. The most serotypes of EPEC are: O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Nataro and Kaper, 1998). From the obtained results, the four serotyping of *E. coli* isolated in this study is following EPEC pathotype as recorded in Table 5. Only two strains of isolated *E. coli* carried *eaeA* virulence gene that has a major role in the attachment of *E. coli* in the epithelium of the intestine by producing intimin protein and this protein can inhibit the absorption capacity of the brush border

Table 2. Statistical analytical results of total coliforms count of the examined milk samples.

Source of the examined samples	No. of examined samples	Positive samples		Count cfu/ ml of the positive sample		
		No.	%	Min.	Max.	Average
Farmer's houses	50	10	20	1.6×10^3	2×10^5	1.9×10^4
Dairy farms	50	3	6	4.6×10^4	8.3×10^4	6.6×10^4
Dairy shops	50	7	14	5.0×10^2	1.6×10^5	4.0×10^4

Table 3. Frequency distribution of the positive examined milk samples based on their total coliforms count.

Interval	Farmer's houses		Dairy farms		Dairy shops	
	No.	%	No.	%	No.	%
10 -	-	0	-	0	-	0
10^2-	-	0	-	0	-	0
10^3-	-	0	-	0	3	6
10^4-	1	2	-	0	-	0
10^5-	5	10	3	6	3	6
10^6-	4	8	-	0	1	2
Total	10	20	3	6	7	14

Table 4. Prevalence of *E. coli* in the examined milk samples.

Source of examined samples	No. of examined samples	No. of positive samples		Egyptian Standard (2010)			
				Compatible		Incompatible	
		No.	%	No.	%	No.	%
Farmer's houses	50	1	2	49	98	1	2
Dairy farms	50	1	2	49	98	1	2
Dairy shops	50	2	4	48	96	2	4
Total	150	4	2.66	146	97.33	4	2.66

Table 5. Serotyping and virulence genes for the isolated *E. coli*.

Source of the examined sample	No. of positive samples	Polyclonal *E. coli*	Monoclonal *E. coli*	Virulence genes	
				eaeA	*hly*
Farmer's houses	1	2	O_{127}:K_{63}	- ve	-ve
Dairy farms	1	2	O_{55}	+ ve	-ve
Dairy shops	2	1	O_{114}:K_{90}	+ ve	-ve
		1	O44 :K74	-ve	-ve

Table 6. Prevalence of *Enterobacter* spp. in the examined milk samples.

Source of examined samples	No. of examined samples	The positive samples of *Enterobacter spp*.	
		No.	%
Farmer's houses	50	2	4
Dairy farms	50	2	2
Dairy shops	50	1	4
Total	150	5	3.33

Table 7. Prevalence of *C. sakazakii* in the examined milk samples.

Source of the examined samples	No. of examined samples	The number of positive samples of *C. sakazakii*	
		No.	%
Farmer's houses	50	7	14
Dairy farms	50	-	0
Dairy shops	50	4	8
Total	150	11	7.33

and stimulation of intestinal secretion (Wilshaw *et al.*, 2000). On the other side, all the isolated strains of *E. coli* didn't carry *hly* A gene which agreed with that postulated by Abd El-Tawab *et al.* (2015); Khatib *et al.* (2015) and Ewida and Hussein (2018).

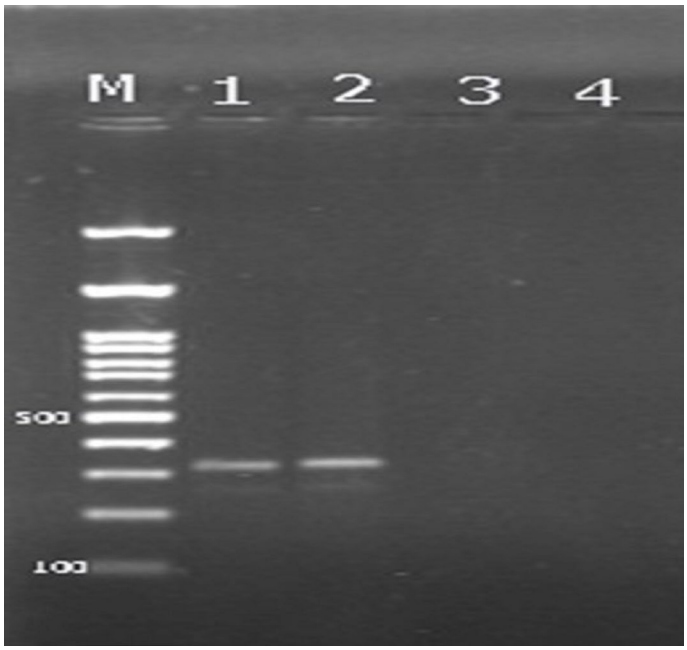

Fig. 1. PCR products of amplified of virulent genes visualized on agarose gel electrophoresis. Lane (M) DNA ladder 100 bp, Lane (1and 2) positive samples for gene *eaeA* at 397 bp, Lane (3 and 4) negative samples for gene *eaeA*.

The obtained percentages of *Enterobacter* spp. (Table 6) are lower than the results pointed out by Elbagory *et al.* (2016); El-Leboudy *et al.* (2017) and Fathi *et al.* (2019). *Enterobacter* spp. Is one of coliforms group, they are ubiquitous and widely distributed in the environment as water, soil and sewage. Moreover, they present in the gastrointestinal tract of humans and animals (Mezzatesta *et al.*, 2012) and cause many infections like pneumonia, meningitis, gastrointestinal infections and urinary tract infection (Locks *et al.*, 2015).

Furthermore, *C. sakazakii* could be detected in the milk samples collected from farmer's houses and dairy shops, while it failed to be detected in milk obtained from dairy farms. The total percentage of isolation was 7.33% (Table 7) which is lower than that demonstrated by Berhilevych and Kasianchuk (2017), and higher than the results detected by Ogihara *et al.* (2019) as they failed to detect *C. sakazakii* in the raw milk samples.

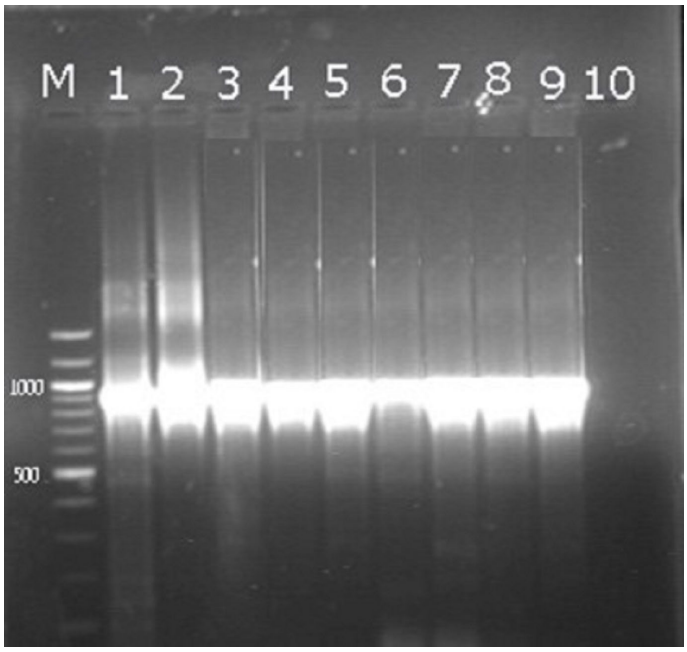

Fig. 2. PCR products of amplified 16S rRNA of *C. sakazakii* visualized on agarose gel electrophoresis. Lane (M) DNA ladder 100 bp, lanes (1-8) positive strains with specific bands at 929 bp, lane (9) positive control and lane (10) negative control.

The presence of *C. sakazakii* in milk may be attributed to contamination from the gastrointestinal tract of humans, animals, insects, and rodents. Fecal-carriage bacteria can survive in the environment for up to 120 days (Molloy *et al.*, 2009).

CONCLUSION

The marketable milk sold in the markets of the New Valley Governorate is contaminated with different coliform species. Certain restrictive preventive measures should be applied during the milking process.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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