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PREVALENCE OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) IN MILK AND SOME DAIRY PRODUCTS

By

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ABSTRACT

Two hundred & eighty samples [100 samples of raw milk, 50 samples of ice cream, 30 samples of plain yogurt and 100 samples of cheese (25 each of soft, hard, processed and Kariesh)] were randomly collected from dairy farms, dairy shops & groceries in Cairo and Giza governorates. Coliforms could be detected in 96%, 58%, 50%, 80%, 84%, 72 % and 60% of raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese samples, respectively. *E. coli* and other species of coliform could be detected in the examined samples at varying percentages. the incidence of Enterohaemoragic *Escherichia coli* by using MUG test were 22.60%, 30%, 25%, 25%, 66.67%, 57.14% and 42.86% of the examined samples of raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese, respectively. Shigtoxigenic *E. coli* could be serologically identified which belonged to O157, O78, O103, O118, O124, O145 and O164, it was evident that shiga toxins could be detected using ELISA test in raw milk, ice cream, Kariesh cheese and processed cheese samples with incidence of 4%, 2%, 4% and 4%, respectively. By using PCR technique, shiga toxin (Stx2) gene was confirmed in 1% of the examined raw milk samples.

<u>Key words:</u>

Shigatoxin producing *E. coli*, coliform, raw milk, dairy products

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is the most important group of food-borne pathogens that emerged recently. The involvement of the pathogenic Shiga-toxin-producing *Escherichia coli* in sporadic cases and outbreaks is presently increasing and it is of most concern in developed countries. Enterohaemoragic *E. coli* (EHEC) or Verocytotoxin producing *E. coli* (VTEC), so called because they produce one or more cytotoxins or shiga toxins that are toxic to Vero cells which similar to cytotoxins produced by Shigella dysenteriae serotype. These bacteria can cause severe health problems in humans like diarrhea, hemorrhagic colitis and hemolytic uraemic syndrome that have become a serious health problem in various countries. Cattle are thought to be a reservoir for STEC. Many foodborne diseases have been associated with the consumption of raw milk and dairy products. **(Mansouri-Najand and Khalili, 2007)**. The term 'EHEC' refers to *E. coli* O157:H7 that is responsible for the greatest proportion of disease cases and non O157 STEC serogroups including O26, O111, O103, O104, O118, O145 (with various H antigen types) and others that share the same clinical, pathogenic and epidemiologic features with *E. coli* O157:H7. **(Riemann and Cliver 2006)**. A number of STEC outbreaks associated with pasteurized milk, cheese, yogurt, ice-cream and milk shakes made with pasteurized milk have been reported. These were probably due to defective pasteurization and/or post processing contamination. **(Becker, 2005; De Schrijver *et al.,* 2008 and Buvens *et al.,* 2011).**

MATERIAL AND METHODS

Two hundred & eighty samples [100 samples of raw milk, 50 samples of ice cream, 30 samples of plain yogurt and 100 samples of cheeses (25 each of soft, hard, processed and Kariesh) were randomly collected under strict sanitary measures from dairy farms, dairy shops and groceries in Cairo and Giza governorates, Egypt. Each sample of raw milk was subjected to Guaiac test **(Schonberg, 1956)** for elimination of those samples proved heat-treated. Preparation of decimal dilutions was adopted according to **APHA (2004).** The incidence of coliforms and *E. coli* were detected using MPN technique as described by **BAM, 2013.** Purified isolates were subjected to further identification according to **Berge's, 2009 and BAM, 2013.** For detection and isolation of Enterohaemoragic *E. coli* (STEC) by cultural isolation on SMA (Sorbitol MacConkey agar) medium and using MUG (4-methyl umbelliferyl-beta-d-glucuronide), methods recommended by **BAM, 2013 and Oxoid, 2010** were applied. *E. coli* isolates were serologically identified by Slide agglutination test for O antigen group screening using *E. coli* antisera, DENKA SEIKEN CO., LTD.Chuo-Ku, Tokyo, Japan, while serological identification of *E. coli* O157 was adopted by latex agglutination test using *E. coli* O157 Latex test kit DR0620 (Oxoid). Qualitative determination of Verotoxins 1 and Shiga-like toxins I and II, of Enterohemorragic *E.* coli was performed using the

RIDASCREEN® Verotoxin test C2201 R-Biopharma AG, Germany (an enzyme immunoassay ELISA kit), detection of the genes necessary for toxin production (Stx1 and Stx2) was carried out using a Polymerase Chain Reaction (PCR) method , conventional PCR relies on amplification of the target gene (s) in a thermocycler, separation of PCR products by gel electrophoresis, followed by visualization and analysis of the resultant electrophoretic patterns which described by **Pollard *et al.,* (1990).**

RESULTS

Table (1): Statistical analytical results of coliform content MPN/ml. or g. in the examined samples.

Type of samples	No. of examined samples	Positive samples		Min.	Max.	Mean ± S.E.M.
		No.	%			
Raw mik	**100**	**96**	**96**	**7 X10^2**	**7.5X10^9**	**3.9X10^8 ± 1.3 X10^8**
Ice cream	**50**	**29**	**58**	**3 X10^3**	**2.3 X10^7**	**2.4X10^6 ± 0.82 X10^6**
Plain yogurt	**30**	**15**	**50**	**30**	**7.5 X10^3**	**7.2X10^2 ± 3.1 X10^2**
Kariesh cheese	**25**	**20**	**80**	**2 X10^3**	**4.6 X10^6**	**5.8X 10^5 ± 2.1 X10^5**
Soft cheese	**25**	**21**	**84**	**7 X10^3**	**7.5 X10^6**	**5.8X10^5 ± 3.1 X10^5**
Hard cheese	**25**	**18**	**72**	**1.1X10^3**	**4.6 X10^6**	**6.3X10^5 ± 2.3 X10^5**
Processed cheese	**25**	**15**	**60**	**1.1X10^4**	**4.6 X10^6**	**5.2X10^5 ± 2.2X10^5**

Table (2): Frequency distribution of coliform content in the examined samples.

Intervals	Raw mik		Ice cream		Plain yogurt		Kariesh cheese		Soft cheese		Hard cheese		Processed cheese	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0-10^2	**4**	**4**	**21**	**42**	**21**	**70**	**5**	**20**	**4**	**16**	**7**	**28**	**10**	**40**
10^2-10^4	**11**	**11**	**1**	**2**	**9**	**30**	**3**	**12**	**1**	**4**	**-**	**-**	**-**	**-**
10^4 - 10^6	**35**	**35**	**14**	**28**	**-**	**-**	**10**	**40**	**16**	**64**	**12**	**48**	**10**	**40**
10^6 - 10^8	**31**	**31**	**14**	**28**	**-**	**-**	**7**	**28**	**4**	**16**	**6**	**24**	**5**	**20**
10^8 - 10^{10}	**19**	**19**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Total	**100**	**100**	**50**	**100**	**30**	**100**	**25**	**100**	**25**	**100**	**25**	**100**	**25**	**100**

Table (3): Incidence of isolated *E. coli* using *MUG test compared with that recovered at 35 ºC and 45.5 ºC.

Type of samples	No. of examined samples	Presumptive positive samples on SMAC		GUD – ve samples	
		No.	%	No.	%
Raw mik	**100**	**31**	**31.00**	**7**	**22.60**
Ice cream	**50**	**10**	**20.00**	**3**	**30.00**
Plain yogurt	**30**	**8**	**26.67**	**2**	**25.00**
Kariesh cheese	**25**	**12**	**48.00**	**3**	**25.00**
Soft cheese	**25**	**6**	**24.00**	**4**	**66.67**
Hard cheese	**25**	**7**	**28.00**	**4**	**57.14**
Processed cheese	**25**	**7**	**28.00**	**3**	**42.86**
Total	**280**	**81**	**28.93**	**26**	**32.1**

Table (4): Detection of *E. coli* O157:H7 isolated from the examined samples using *SMAC and MUG.

Type of samples	No. of examined samples	Incidence of *E.coli* at 35ºC				Incidence of *E.coli* at 45.5ºC			
		E.coli positive samples		*GUD +ve		*E.coli* positive samples		GUD +ve	
		No.	%	No.	%	No.	%	No.	%
Raw mik	**100**	**15**	**15.63**	**4**	**26.67**	**10**	**10.00**	**4**	**40.00**
Ice cream	**50**	**5**	**17.24**	**1**	**20.00**	**4**	**8.00**	**1**	**25.00**
Plain yogurt	**30**	**1**	**6.67**	**1**	**100.00**	**1**	**3.33**	**1**	**100.00**
Kariesh cheese	**25**	**7**	**35.00**	**2**	**28.57**	**4**	**16.00**	**2**	**50.00**
Soft cheese	**25**	**6**	**28.57**	**2**	**33.33**	**3**	**12.00**	**2**	**66.67**
Hard cheese	**25**	**4**	**22.22**	**1**	**25.00**	**1**	**4.00**	**1**	**100.00**
Processed cheese	**25**	**1**	**6.67**	**1**	**100.00**	**1**	**4.00**	**1**	**100.00**
Total	**280**	**39**	**14.39**	**12**	**30.77**	**24**	**8.57**	**12**	**50.00**

*** MUG: 4-methyl umbelliferyl-beta-d-glucuronide.**

*** GUD: Glucouronidase enzyme.**

*** SMAC: Sorbitol MacConkey Agar.**

Table (5): Serological identification of suspected *E.coli* isolates by using slide agglutination test.

Serotypes	Raw milk		Ice cream		Plain yogurt		Kariesh cheese		Soft cheese		Hard cheese		Processed cheese	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
O78	1	1.00	-	-	1	3.33	-	-	-	-	1	4.00	-	-
O103	-	-	-	-	-	-	1	4.00	-	-	-	-	1	4.00
O118	1	1.00	-	-	-	-	-	-	-	-	-	-		
O124	-	-	-	-	-	-	1	4.00	-	-	-	-	-	-
O145	1	1.00	1	2.00	-	-	-	-	-	-	-	-	-	-
O157	1	1.00	-	-	-	-	-	-	1	4.00	-	-	-	-
O164	-	-	-	-	-	-	-	-	1	4.00	-	-	-	-
Total	4	4.00	1	2.00	1	3.33	2	8.00	2	8.00	1	4	1	4

Table (6): Incidence of isolated shiga toxigenic *E.coli* by using ELISA and PCR tests.

Type of samples	No. of examined samples	Serologically +ve samples		Shiga toxins +ve samples using ELISA		Shiga toxins +ve samples using PCR	
		No.	%	No.	%	No.	%
Raw milk	100	4	4.00	4	4.00	1	1.00
Ice cream	50	1	2.00	1	2.00	-	-
Plain yogurt	30	1	3.33	-	-	-	-
Kariesh cheese	25	2	8.00	1	4.00	-	-
Soft cheese	25	2	8.00	-	-	-	-
Hard cheese	25	1	4.00	-	-	-	-
Processed cheese	25	1	4.00	1	4.00	-	-
Total	280	12	49.33	7	14.00	1	1.00

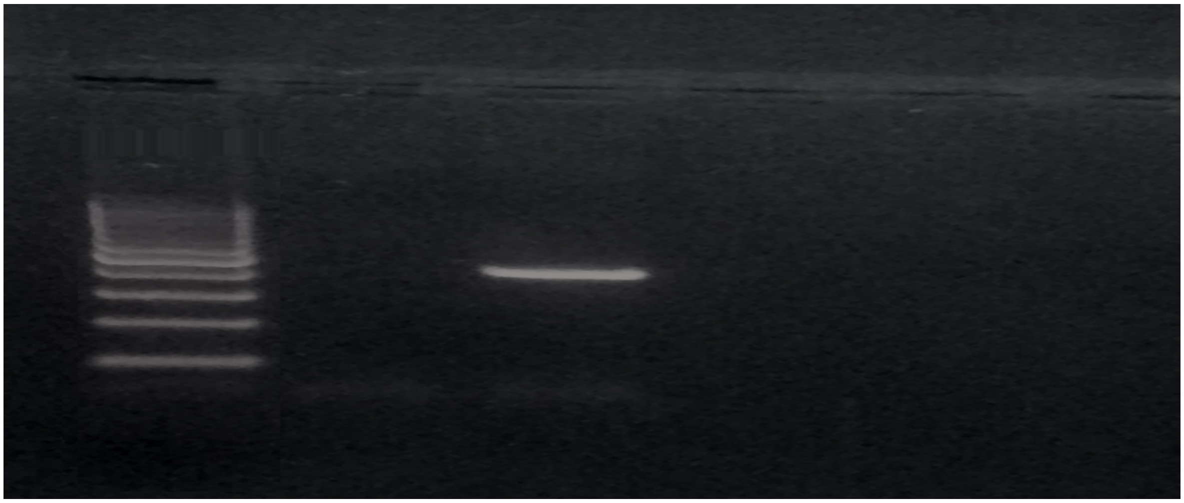

Photo (1): Electrophoretic analysis on 1.5 % agarose gel stained with ethidium bromide.
Lane 1: Molecular weight Marker (100bp ladder).
Target gene: Sequence (5′ to 3′)
Stx1: AGTTAATGTGGTGGCGAA (817 bp)
GACTCTTCCATCTGCCG
Stx2: TTCGGTATCCTATTCCCG (474 bp)
TCTCTGGTCATTGTATTA
Lane 3: PCR product of *E. coli* positive for Stx 2 gene.

DISCUSSION

Coliform count is the traditional indicator of possible fecal contamination, microbial quality and wholesomeness, which reflect the hygienic standards adopted in the food operation. Lack of good sanitary practices may result in loss of quality, spoilage or, in some cases, create a health hazard **(Vanderzant and Splittstoesser, 2005)**. Regarding the results represented in Table 1, coliform were detected in 96 (96%), 29 (58%), 15 (50%), 20 (80%), 21 (84%),18 (72%) and 15 (60%) of raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese samples, respectively, with mean values of $3.9X10^8 \pm 1.3 X10^8$, $2.4X10^6 \pm 8.2 X10^5$, $7.2X10^2 \pm 3.1 X10^2$, $5.8X 10^5 \pm 2.1 X10^5$, $5.8X10^5 \pm 3.1 X10^5$, $6.3X10^5 \pm 2.3 X10^5$ and $5.2X10^5 \pm 2.2X10^5$, respectively. The highest frequency of positive samples in ice cream was 42 % and in plain yogurt was 70% lies within the range of (10^2-10^4). While the highest frequency of positive samples in raw milk (35%), Kariesh cheese (40 %), soft cheese (64%) and hard cheese (48%) lies within the range of (10^4 - 10^6). 40 % of positive samples in processed cheese lies within the range of (0-10^2) and (10^4 - 10^6) (Table 2). **De-Azevêdo *et al.* (2014) obtained** nearly similar results of raw milk**. Ibrahim *et al.* (2015)** recorded higher results**,** while **Nwankwo *et al.* (2015)** detected lower results. High incidence of coliform in the examined raw milk samples indicated neglected sanitary conditions that could be due to infected udders, unhygienic milking procedures or equipments, and or inferior microbiological quality of water used for cleaning of utensils and animals as well as the milk storage conditions. Therefore, poor milk quality has been considered as one of the major reasons for losses and reduction of income **(Donkor *et al.,* 2007)**. **Hadrya *et al.* (2012)** detected nearly similar findings of coliforms in ice cream**. Karim and Dey (2013)** obtained higher results**,** while **Gürler *et al.* (2013)** recorded lower results. The presence of coliforms in ice cream samples indicated the poor hygienic practices during manufacture, post-processing contamination and unsatisfactory transportation **(El-Ansary, 2015)**. **Mohamed (2013) and El-Ansary (2014)** obtained nearly similar results of yogurt. **El-Leboudy *et al.* (2015**) recorded higher results, while **El-Malt *et al.* (2013**) obtained lower results. Lower counts of coliform organisms in the examined samples of plain yogurt indicated good manufacturing practices together with the defensive nature of these products such as high acidity and production of antimicrobial substances (bacteriocins) by yogurt starter culture **(Tamime and Robinson, 1999)**. **El-Leboudy *et al.* (2015)** obtained nearly similar results of Kariesh cheese**,** while **Ibrahim *et al.* (2015)** recorded higher results. **Fahim (2012**) obtained nearly similar findings of soft cheese**. Ibrahim *et al.* (2015)** detected higher results**,** while lowere results were obtained by **El-Sayed *et al.* (2011) and Brooks *et al.* (2012)**. Nearly similar results of hard cheese and processed cheese were obtained by **Abo Zeed (2014) and El-Leboudy *et al.* (2015).**Contamination of cheese samples with coliforms gives indication of bad hygienic conditions during production, handling and distribution **(Jafar and Jalil, 2012)**. The International Commission on Microbiological Specifications for Foods has classified cheese and ice cream as a high-risk potential hazard may be due to their nutrient content, long storage duration and several steps in their production can cause bacteriological hazards, this could be confirmed by their implication in several outbreaks of food poisoning. It is implicated in gastrointestinal illness as gastroenteritis, epidemic diarrhea in children and cases of food poisoning **(Quinto and Cepeda, 1997)**. High coliform content in food products render them of inferior quality and became unmarketable during storage or even unfit for human consumption causing economic losses **(ICMSF, 1996)**. It is indicative of post pasteurization contamination at one or more stages during processing **(Ekram and Ibtisam, 2011)**.

Incidence of E. coli

Fecal contaminations of foods during preparation are the most common source of Enteropathogenic *E. coli.* Diarrhea genic *E. coli* strains are diverse food-borne pathogens and causes diarrhea with varying virulence in humans **(Caine *et al.,* 2014)**. Results presented in (Table 3) showed the incidence of *E coli* recovered at 35°C and 45.5°C in raw milk (15.63% & 10.00%), ice cream (17.24% & 8.00%), plain yogurt (6.67% & 3.33%), Kariesh cheese (35.00% and 16.00%), soft cheese (28.57% & 12.00%), hard cheese (22.22% & 4.00%) and processed cheese (6.67% & 4.00), respectively. **Nwankwo *et al.* (2015) and Ombarak *et al.* (2016) obtained** higher results of raw milk. **Ahmed *et al.* (2009)** obtained nearly similar results of ice cream**. Virpari *et al.* (2013**) obtained higher results**,** while **Baraheem *et al.* (2007) and Ghasemi *et al.* (2009)** detected lower results**. Okpalugo *et al.* (2008) and Virpari *et al.* (2013)** detected higher findings of yogurt**. Ibrahim *et al.* (2015)** obtained higher results of Kariesh cheese**.** While **Nosir *et al.* (2014) and Ombarak *et al.* (2016) obtained** lower results**. Walaa Hassan (2008)** obtained lower results of soft cheese, while **Sharaf *et al.* (2014) and Ibrahim *et al.* (2015)** recorded higher results. **Nosir *et al.* (2014) and Ombarak *et al.* (2016) obtained** higher results of hard cheese**. Fahim (2012)** recorded lower results of processed cheese. The sensitivity of MUG test based on GUD (Glucouronidase enzyme) for the detection of pathogenic *E. coli*, showed that *E. coli* recovered at 35 °C and 45.5 °C were (26.67% & 40%), (20% & 25%), (100% &100%), (28.57% &50%), (33.33% & 66.67%), (25% & 100%) and (100% & 100%), of raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese samples, respectively. MUG (4-methyl umbelliferyl-beta-d-glucuronide) is employed in plating media; it is the most widely used flurogenic substrate, it is hydrolyzed by ß-Glucouronidase (GUD) which produced by pathogenic *E. coli* to release the fluorescent 4-methyl umbelliferon, which is detected with long wave ultraviolet light 366 nm. **(Essa, 2000)**. The mean values at 45.5 °C were lower than that recorded at 35 °C. A result that reflects the inhibitory effect of incubation temperature on different *E. coli* rather than pathogenic ones **(Essa, 2000)**. The occurrence of high proportion of *E. coli* in the examined samples may be due to lack of proper sanitation and absence of pasteurization of milk used for processing of dairy products **(Mansuri-Najand and Ghanbarpour, 2006)**. *E. coli* O157:H7 seems to have no particular tolerance to salt levels; bacterial growth has been documented with Nacl concentration ranging from 2.5 to 6.5%, when other growth-affecting factors were favorable **(Conner, 1992).**

Verocytotoxin producing *E. coli* O157:H7:

Verocytotoxin producing *E. coli* (VTEC), particularly *E. coli* O157:H7 organisms are responsible for a variety of conditions ranged from aqueous diarrhea, through hemorrhagic colitis to hemolytic uremic syndrome and thrombotic thrombocytopenic purpura in infected individuals, and may be fatal in children and elderly. (Table 4) showed that, the incidence of *E. coli* O157:H7 on SMAC (sorbitol MacConkey agar) were 31%, 20%, 26.67%, 48%, 24%, 28% and 28% of the examined samples of raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese, respectively, while those detected by using MUG were 22.60%, 30%, 25%, 25%, 66.67%, 57.14% and 42.86% of the examined samples, respectively. **Anklam *et al.* (2012); Ivbade *et al.* (2014) and Enem *et al.* (2015)** obtained nearly similar results. The enterohemorrhagic *E. coli* O157:H7 recently considered as a major public health problem, as it caused an estimated 20,000 infections and 250 deaths per year in United States and many outbreaks have been associated with the consumption of dairy products **(Djuretic *et al.*, 1997 and Dardir, 1999).**

Serological identification of suspected E.coli isolates

Serotyping is effective in revealing some of the diversity among *E. coli* and is often the starting point in characterization of *E. coli*. It is based on the highly immunogenic variable lipopolysaccharides (LPS) presented on the bacterial cell surface of which about 200 forms are found in *E. coli* **(Gyles, 2007)**. The results of *E. coli* serogroups illustrated in (Table 5) showed that; 4 (4%) isolates from raw milk samples serotyped as O78, O118, O145 and O157 with an incidence of 1%, 1%, 1% and 1%, respectively, while *E. coli* strains isolated from ice cream and plain yogurt samples serotyped as O145 and O78 with incidence of 1% and 1%, respectively. The isolated *E. coli* strains from Kariesh cheese samples serotyped as O103 (4%) and O124 (4%), while those isolated from the examined soft cheese samples were serotyped as O157 (4%) and O164 (4%). The isolated *E. coli* strains from the examined samples of hard and processed cheese serotyped as O78 (4%) and O103 (4%), respectively. **Ahmed and Shimamoto (2014)** obtained nearly similar results of raw milk**,** while **Lingathurai and Vellathurai (2010)** recorded higher results **and Ivbade *et al.* (2014)**. **Rahimi *et al.* (2011)** recorded nearly similar results of ice cream**,** while **Momtaz *et al.* (2012) and Virpari *et al.* (2013)** obtained higher results**. Rahimi *et al.* (2011)** detected nearly similar results of yogurt. **Ahmed, Esraa (2012),** detected nearly similar results of Kariesh cheese while **Ibrahim *et al.* (2015)** obtained higher results. **Madic *et al.* (2011)** recorded nearly similar findings of soft cheese. **Ahmed, Esraa (2012)**, detected lower results while **Momtaz *et al.* (2012**) obtained higher results.The isolated serotypes are of public health concern, as they have been associated with several outbreaks. The earliest reported outbreak caused by serotype O145: H-, occurred in **Japan in 1984 (Karmali *et al.,* 1985)**. Verocytotoxin-producing *E. coli* O157 (VTEC/STEC) comprise over 400 serotypes. Serotype O157:H7 is prevalent in many world regions and considered the most dangerous vegetative pathogen associated with raw milk and milk products **(Baylis, 2009).**

Detection of shiga toxin producing E. coli using ELISA and Polymerase Chain Reaction (PCR):

PCR and ELISA tests were highly specific, sensitive and rapid for detecting Shiga toxin producing Escherichia coli (STEC), and their prevalence in dairy products **(Fach *et al.,* 2001).** Regarding the results represented in (Table 6), it was obvious that the pathogenic *E. coli* serotypes were detected in 4%, 2%, 3.33%, 8%, 8%, 4% and 4% of the examined raw milk, ice cream, plain yogurt, Kariesh cheese, soft cheese, hard cheese and processed cheese samples, respectively. It was evident that shiga toxins could be detected using ELISA test in raw milk, ice cream, Kariesh cheese and processed cheese samples with incidence of 4%, 2%, 4% and 4%, respectively, while it could not be detected in the examined samples of plain yogurt, Kariesh cheese, soft cheese and hard cheese. By using PCR technique, shiga toxin (Stx2) gene was confirmed in 1% of the examined raw milk samples. With the inspection of PCR results presented in photo (1), the electrophoretic analysis of PCR of shigatoxin producing *E. coli* O157:H7 on 1.5% agarose gel stained with ethidium bromide showed positive band for Stx2 producing *E. coli* strain isolated from raw milk samples. Nearly similar results using ELISA were recorded by **Enem *et al.* (2015),** while lower findings were obtained by **Timm *et al.* (2009) and Kiranmayi and Krishnaiah (2011). Zweifel *et al.* (2010) and Mohammadi *et al.* (2010) obtained** Higher results were obtained by**. Ivabade *et al.* (2014)**, while **Virpari *et al.* (2013) obtained higher results and Kalmus *et al.* (2015)** detected nearly similar findings using PCR**; Caine *et al.* (2014)** obtained lower results**.** Low incidence of STEC in the examined samples may be due to the presence of high levels of competing microflora or of natural inhibitors within dairy products that interfere with STEC isolation. STEC might also be present in a stressed or injured state that prevents its isolation **(Madic *et al.,* 2011).** Milk -borne transmission of Shiga toxin- producing *Escherichia coli* (STEC) has raised considerable concern due to recent outbreaks worldwide and poses a threat to public health **(Vendramin *et al.,* 2014)**. The common feature and main virulence factor of STEC is the production of Shiga toxin 1 (**Stx1**), Shiga toxin 2 (**Stx2**) or its variants, or a combination of these. **(Paton and Paton, 1998)**.

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