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STUDIES ON SOME MILK BORNE PATHOGENS

SAADIA H.H. EL-SHINAWY [1]; GAMAL M. HASSAN [1]; MOHAMED A.H. El-SHATER [2] and SHIMAA A. ABD El-AZIZ [2]

[1] Food Hygiene Department, Faculty of Veterinary Medicine, Beni-Suef University
[2] Animal Health Research Institute, Egypt

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ABSTRACT

The objective of this study was to assess the degree of contamination of raw and pasteurized milk with bacteria of the genus *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli O157: H7*. A total of 200 milk samples (150 raw and 50 pasteurized) were purchased from different sources; farms, collecting centers, dairy shops and different super markets in El-Minia governorate, Egypt. The samples were examined bacteriologically and presumptive isolates were further confirmed by PCR using specific primers of *Staph. aureus* clfA gene, *L. monocytogenes* 16S rRNA gene and serotyping of *E. coli* isolates. The overall incidence of *Staph. aureus* was 64% and 4% in raw and pasteurized milk, respectively, while *L. monocytogenes* was present in 8% of raw milk and couldn't be detected in pasteurized milk. Moreover, *E. coli O157: H7* could not be detected in any samples. In conclusion, raw milk represents a public health hazard for human consumption in relation to milk borne pathogens.

Key words: Raw milk, Pasteurized milk, *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli O157:H7,* Public health.

INTRODUCTION

Since ancient times, milk form a major part of human food and play a prominent role in the diet (Pal, 2014). Raw milk is known as the main transmission pathway for pathogens resulting in food borne outbreaks every year (Gillespie *et al*., 2003; Rey *et al*., 2003). Pasteurization process of milk is a heat treatment intended to reduce the number of any harmful microorganisms to a level at which they do not constitute a significant health hazard (MPI NZ, 2013).

Staphylococcus. aureus is one of the major bacterial agents causing food borne diseases in human worldwide (EFSA, 2010). It is an opportunistic pathogen, which associated with food poisoning and food spoilage (Argudin *et al.,* 2010). The staphylococcus food poisoning is a mild intoxication occurring after ingestion of food containing from 20ng up to 1 ug of Staphylococcal enterotoxins (SEs) which is enough to induce symptoms in human beings (Nermano *et al.,* 2007). Clinical signs of Staphylococcal food poisoning generally disappear within 24-48 h. Deaths occur rarely and specifically in the young or elderly (Jay *et al.,* 2005).

Corresponding author: Dr. SHIMAA A. ABD El-AZIZ
E-mail address: shimaa_ahmed1415@yahoo.com
Present address: Animal Health Research Institute, Egypt

Listeria monocytogenes is considered as one of the most important food borne pathogen that induce serious and potentially life threatening illness known as listeriosis in humans and animals (Rahimi *et al*., 2012) with high mortality rate (20-30%) (Wijendra *et al*., 2014). It may range from non -invasive febrile gastroenteritis or influenza like symptoms especially in healthy individuals with no predisposing conditions (Aygun and Pehlivanlar, 2006) to serious invasive severe symptoms which may lead to septicemia, meningitis and abortion (Shamloo *et al*., 2015; Kevenk and Gulel, 2016).

Enterohemorrhagic *Escherichia coli* (EHEC) have emerged as an important cause of human intestinal diseases in developed countries over the past 20 years (Naylor *et al*., 2003). *E.coli O157:H7* causes both outbreaks of diarrhea, haemorrhagic colitis and haemorragic uremic syndrome (HUS) (Baker *et al*., 2007). The infectious dose was very low about 100-200 organisms or even less than 10 cells in susceptible consumers which is hazardous and increased the risk of disease (CFSPH, 2009; Grant *et al.,* 2011)**.** In addition, its ability to produce Shiga toxins (*Stxs)* (formerly called Shiga-like toxins) (Beneduce *et al.,* 2003) which is considered as best essential virulence factor for *E. coli O157:H7* in human disease (Mauro and Koudelka, 2011).

Therefore, the present study aimed to study the incidence of some foodborne pathogens, as *Staph.*

aureus, L. monocytogenes and *E.coli O157:H7* in raw and pasteurized milk in El-Minia governorate with regarding to public health.

MATERIALS AND METHODS

1. Collection of Samples:

a-Raw milk: One hundred and fifty raw milk samples were purchased from 3 different sources as farms, dairy shops and collecting centers (50 each) in El-Minia governorate. All samples were collected in clean, dry, sterile containers and delivered as soon as possible to the laboratory in an insulated ice box, proved to be raw by storch's test according to the method recommended by FDA (1998) and examined at the same day.

b- Pasteurized milk: Fifty samples of pasteurized milk (bottles or cartons) were collected from different supermarkets and shops of different manufacture dates and different companies in El-Minia governorate. All bottles and cartons were thoroughly cleaned from outside and then well mixed and aseptically opened.

2- Enumeration, Isolation and identification of *Staph. aureus*:

By the using of Baird Parker's agar plates supplemented with egg yolk and potassium tellurite, the method recommended by APHA (2004) was applied.

3- Isolation and identification of *L. monocytogenes*:

By the using of PALCAM agar, the plates were incubated at 35°C for 48 hours according to Hitchins (2001).

4- Isolation and identification of *E. coli O157 H7*:

By the using of cefixime tellurite-sorbitol MacConkey medium, the incubation was at 37°C for 24 hrs ±2 hrs according to De Boor and Heuvelink (2000).

5- Serological confirmation of the *E. coli* isolates:

All the suspected isolates were serologically identified by slide agglutination according to Kok *et al.* (1996) by using rapid diagnostic *E.coli* antisera sets (DENKA SEIKEN Co., Japan), non sorbitol fermenting (NSF) colonies were subjected to agglutination test by using monvalent O157and H7 antisera (Oxoid).

6- Molecular characterization of L. monocytogenes and Staph. aureus by PCR:

6.1. Extraction of DNA for PCR:

DNA was extracted from colonies by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) Catalogue no.51304 according to the manufacturer's instructions.

6.2. DNA amplification:

Conventional PCR was carried out for identification of the DNA extracts to confirm the presence of genetic material of genus *Staph.aureus* and *L.monocytogenes*. Amplification of target genes (*clfA* and *16S rRNA*) respectively was carried out in a thermal cycler (Biometra, Germany) using 25µl reaction volume containing (12.5 Emerald Amp GT PCR mastermix (2x premix), 5.5 µl PCR grade water, 1 µl Forward primer (20 pmol), 1 µl Reverse primer (20 pmol) and 5 µl Template DNA.

Oligonucleotide primers sequences.

Microbial agent	Target gene	Oligonucleotide Sequence (5\ – 3\)	Amplified product size (pb)	Reference
L. monocytogenes	*16S rRNA*	GGACCGGGGCTAATA CCGAATGAT AA TTCATGTAGGCGAGTT GCAGCCT A	1200 bp	Kumar *et al.* (2015)
Staph. aureus	*ClfA*	GCAAAATCCAGCACAA CAGGAAACGA CTTGATCTCCAGCCAT AATTGGTGG	638 bp	Mason *et al.* (2001)

Cycling conditions of the different primers during cPCR

Microbial agent	Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No.of cycles	Final extension
L.monocytegenes	*16S rRNA*	94˚C 5 min.	94˚C 30 sec.	60˚C 1 min.	72˚C 1 min.	35	72˚C 12 min.
Staph. aureus	*clfA*	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.

6.3. Detection and identification of PCR product.
The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5 μg/ml) and the gels can be screened and pictured under UV light.

RESULTS

Table 1: Incidence of *Staph .aureus* in the examined raw milk samples.

The samples origin	No. of the examined samples	Positive samples	
		No.	%
Farms	50	45	90%
Collecting Centers	50	24	48%
Dairy shops	50	27	54%
Total	150	96	64%

Table 2 : Statistical analytical results of *Staph. aureus* count/ml in the examined milk samples.

The samples origin	No. of examined samples	Minimum	Maximum	Mean ± SEM
Farms	50	2.2×10^2	2.68×10^4	$5.45\times10^3 \pm 1.15\times10^3$
Collecting centers	50	2×10	1.06×10^4	$1.80\times10^3 \pm 3.96\times10^2$
Dairy shops	50	8×10	1.5×10^6	$9.78\times10^4 \pm 5.06\times10^4$
Pasteurized milk	50	30	60	1.8±1.3

Table3: Frequency distribution of the positive samples based on their *Staph. aureus* count/ml.

Intervals cfu/ml	Raw milk						Pasteurized milk	
	Farm		Collecting centers		Dairy Shops			
	No.	%	No.	%	No.	%	No.	%
10-<10^2	0	0	4	16.7	2	7.4	2	100
10^2-<10^3	31	68.9	9	37.5	5	18.5	0	0
10^3-<10^4	4	8.9	9	37.5	10	37.1	0	0
10^4-<10^5	10	22.2	2	8.3	8	29.6	0	0
>10^5	0	0	0	0	2	7.4	0	0
Total	45	100%	24	100%	27	100%	2	100%

Table 4: Acceptable and unacceptable samples of the examined milk samples based on their *Staph. aureus* count/ml. according to the Egyptain standards (2005).

Source of the samples	Permissible limit	Acceptable samples		Unacceptable samples	
		No.	%	No.	%
Farm	Must be not more than 10^2	5	10	45	90
Collecting centers	Must be not more than 10^2	30	60	20	40
Dairy shops	Must be not more than 10^2	25	50	25	50
Pasteurized milk	Must be 0	48	96	2	4

Table 5: Incidence of *L. monocytogenes* in the examined raw milk samples.

The samples origin	No. of examined samples	Positive samples	
		No.	%
Farms	50	7	14%
Collecting Centers	50	2	4%
Dairy shops	50	3	6%
Total	150	12	8%

Table 6: Some *E.coli* serotypes isolated from the examined raw milk samples.

The samples origin	Poly Valent	Mono Valent
Farms	3	O114
	3	O142
Dairy shops	1	O86a
	2	O146

Table 7: Incidence of *Staph .aureus, L. monocytogenes and E. coli O157:H7* in the examined pasteurized milk samples.

No. of the examined samples	*Staph.aureus*		*L. monocytogenes*		*E. coli O157:H7*	
50	No.	%	No.	%	No.	%
	2	4	0	0	0	0

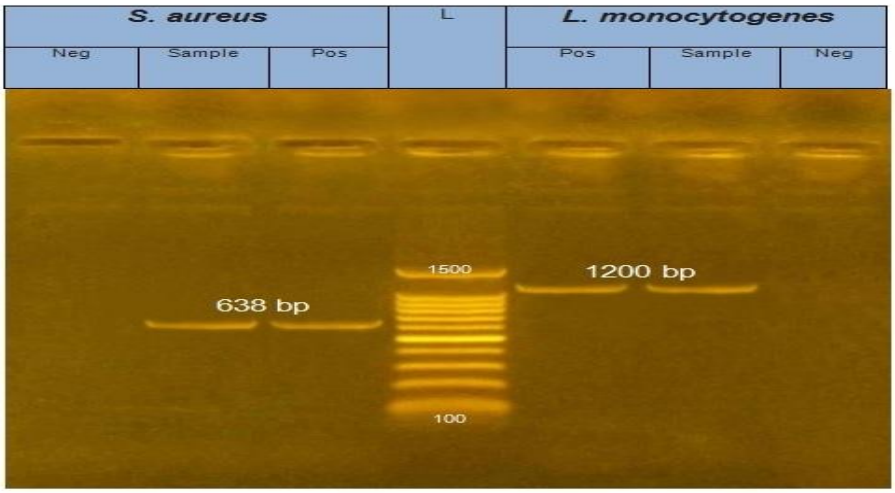

Photo1: PCR results for *Staph. aureus* clfA gene and *L. monocytogenes* 16S rRNA gene showing positive amplification 638 bp of *Staph. aureus* clfA gene and 1200 bp of *L. monocytogenes* 16S rRNA gene, L showed [Gelpilot100 bp plus ladder (Qiagen, 100-1500 bp)].

DISCUSSION

The results illustrated in Table 1 revealed that, 96 (64%) of the examined raw milk samples were contaminated with *Staph. aureus.* Nearly similar findings 52% and 56.66% was reported by Pourhassan and Taravat (2011); El-Jakee *et al.* (2013), respectively. However, higher results of *Staph.aureus* isolated from cow's milk reported by Al-Tarazi *et al.* (2003); Ekici *et al.* (2004) in percentages of 80 and 75 % respectively. While, lower results were estimated by Jahan *et al.* (2015); Ayele *et al.* (2017) in percentages 25.3 and 23.4% respectively.

Staph. aureus are wide spread in nature, up to 50% of humans may carry this organism in their nasal passages, throats and on their hair and skin. So it is

good indicator of the personal hygiene of the workers with respiratory infections (Kamat *et al*., 1991; Harvey and Gilmour, 1996).

The results in Table 4 proved that 10%, 60%, 50% and 96% of the milk samples from farms, collecting centers, dairy shops and pasteurized milk respectively were acceptable for the *Staph. aureus* cfu/ml according to the Egyptian standards (2005).

The obtained results reported in Table 5 reveals that raw milk was contaminated with *L. monocytogenes* with an overall incidence of 8%. Nearly similar results were recorded by El-Malt and Abdel–Hameed (2009); El-Marnissi *et al.* (2013); Kevenk and Gulel (2016). Meanwhile, these results disagreed with those recorded by Sammarco *et al* (2005); Aygun and Pehlivanlar (2006); Atil *et al*. (2011) who failed to isolate *L. monocytogenes*.

The sources of Listeria spp. in raw milk have been reported to be faecal (Griffiths, 1989) and environmental contamination during milking, storage and transport, infected cows in dairy farms and poor silage quality (Bemrah *et al*., 1998). High contamination of milk with L. monocytogenes in autumn and winter when silages are fed. Broseta *et al*. (2003) also reported that contamination of raw milk with *L. monocytogenes* is usually more common in the winter, most likely because silage feeding in many parts of the world is more common in that season. In addition, a large number of studies have indicated that clinical listeriosis in ruminants is often associated with feeding poor quality silage (Boerlin *et al*., 2002).

Silage is not widely used as animal feed in El-Minia governorate, Egypt.; the animals were fed with dry feed and some green grass. Therefore, the contamination source of *L. monocytogenes* in raw milk in this study may be of fecal and environmental contamination during milking, storage and transport, infected animals in dairy farms**.** Furthermore, most cattle and sheep farms in Egypt do not have adequate hygiene precautions and animals live in a natural environment together with people.

E.coli O157: H7 could not be detected in all of the examined samples using conventional culture, biochemical and serological methods. Serological identification of the isolated *E. coli* are listed in Table 6 where serotypes O114, O142, O86a and O 148 were detected.

The obtained results was in line with the results obtained by Coia *et al.* (2001); Dontorou *et al.* (2003); Meshref (2007); Issa *et al.* (2010); Zeinhom (2011) who couldn't isolate *E. coli O157 H7* from raw milk examined in Netherlands, Greece, Turkey and Egypt respectively. On the contrary the current results disagreed with others like Abdel Khalek *et al.* (2001); Amer and Soliman (2004); El-Gedawy *et al.* (2016) who reported 3, 1 and 1 % of raw milk examined in Egypt were contaminated with E. coli O157:H7.

The failure in detection of *E. coli O157:H7* in milk is mainly returned to its presence sporadically at very low levels among very high levels of competitor organisms (Siriken *et al.*, 2006). It can be thought that there is very low risk, but on the other hand, this bacterium can survive for more than 50 days in municipal reservoir and lake water (Wang and Doyle, 1998) and dairy cattle are asymptomatic carriers of this bacterium (Zhao *et al*., 1995; Wang *et al*., 1997; Heuvelink *et al.*, 1998), thus increasing the risk for transmission through cattle to cattle, milk and milk products and to other foods.

As recorded in Table 7 it was apparent that out of the 50 examined samples of pasteurized milk, Staph. aureus was isolated from 2 samples (4%). This result was similar to that obtained by Vahedi *et al.* (2013). While higher percentages were recorded by Gu¨ndog˘an *et al.* (2006); Rall *et al.* (2008); De Oliveira *et al.* (2011). Unlike, Leite *et al.* (2002); Ayele *et al.* (2017) did not detect Staph. aureus in the pasteurized milk in Salvador and Ethiopia respectively.

The presence of *Staph. aureus* in pasteurized samples can be explained either by post-pasteurization contamination or by the presence of heat resistant strain. When heat treatment process is ineffective; faulty pasteurization will not destroy all foodborne pathogens (Kadariya *et al*., 2014). Insufficiently cleaned milk equipment was the most frequently incriminated source of pasteurized milk contamination with *Staph. aureus*.

It was clear from the data showed in Table 7 that L. monocytogenes could not be detected in all of the examined samples. These results are in agreement with those obtained by Baek *et al.* (2000); Padilha *et al.* (2001); Abd El Aal and Atta (2009). On the contrary, the current results disagreed with others like, Ahmed and Hussein (2005); Seyoum *et al.* (2015) as they reported 4 and 1 % of pasteurized milk examined were contaminated with L. monocytogenes.

Similarly *E. coli O157:H7* couldn't be detected in pasteurized milk, unlike Upton and Coia (1994) who could isolated it after occurring an outbreak of *E. coli O157* infection associated with pasteurized milk supply. This outbreak resulted in 9 children developing haemolytic uraemic syndrome (HUS) and one elderly woman developing thrombotic thrombocytopenia purpura (TTP).

It was concluded that, some milk borne pathogens like *Staph. aureus* and *L. monocytogenes* could be isolated from raw milk, in which it is unsafe for human consumption.

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دراسات علي بعض مسببات الأمراض في اللبن

سعديه حلمي الشناوي ، جمال محمد حسن ، محمد احمد حسن الشاطر ، شيماء احمد عبد العزيز

E-mail: shimaa_ahmed1415@yahoo.com Assiut University web-site: www.aun.edu.eg

أجريت هذه الدراسة علي ٢٠٠ عينة من اللبن وتشتمل على ١٥٠عينة من اللبن الخام تم تجميعها من المزارع ، محلات بيع الألبان ومراكز تجميع الألبان و٥٠ عينه من اللبن المبستر تم تجميعها من المحلات المختلفة بمحافظة المنيا. وإشتملت الدراسة علي فحص هذه العينات لتواجد ميكروب المكور العنقودي الذهبي ، الليستريا مونوسيتوجينز والإيشيريشيا كولاي O157:H7 وأسفرت النتائج عن تواجد ميكروب المكور العنقودي الذهبي في العينات المفحوصة بنسبة ٦٤% في اللبن الخام و ٤% في اللبن المبستر. كما تم عزل ميكروب الليستريا مونوسيتوجينز بنسبة ٨% من اللبن الخام ولم يتم عزله تماما من اللبن المبستر. كما أوضحت الدراسة أنه لم يتم عزل ميكروب الإيشيريشيا كولاي O157:H7 من كل من اللبن الخام والمبستر. وقد تم مناقشة هذه النتائج التي تشيرإلى وجود تأثير ضار علي الصحة العامة من إستهلاك اللبن الخام ويجب إتخاذ الطرق المناسبة للحد من تلوث اللبن الخام والمبستر بهذه الميكروبات الممرضة.