MOLECULAR IDENTIFICATION OF SOME CONTAGIOUS MICROORGANISMS CAUSING FOOD POISONING FROM BULK TANK MILK IN GHARBIA GOVERNORATE

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A B S T R A C T

This study was conducted to determine the prevalence of food poisoning pathogens in Bulk tank milk from 3 dairy herds in Gharbia Governorate, Egypt. *Escherichia coli, Staphylococcal aureus* and *Listeria monocytogenes* were detected in 20, 60, and 10 % in samples collected from farm I , in farm II with percentage of 40, 40 and 20 % and in farm III 20, 20 and 10 % of examined bulk tank milk samples, respectively. Polymerase chain reaction is a powerful technique for detection of pathogens in foods. It is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources. The eight *E. coli* isolates were screened for the presence of virulence associated genes (*stx1, stx2*), heat-stable enterotoxin gene (*STa*) and only one (50%) isolate from farm I encoded the *STa* gene. The ability of *Staphylococcus aureus* to produce enterotoxins which is linked to *Staphylococci enterotoxins SEs* genes was investigated by using multiplex PCR, out of 12 *Staph. aureus* isolated from the examined BTM samples, 2 isolates were carrying sea gene, 1(16.6%) from farm I and 1(25%) from farm II. *Listeria monocytogenes* detection methods based on PCR amplification of the *hly* gene sequences specific for confirmation of *L. monocytogenes* and not any other type of *Listeria* have been used for identification of all four obtained isolates and the results obtained from isolation were in line with that of molecular diagnosis as PCR detected only the presence of *L. monocytogenes*. Since presence of these food poisoning microorganisms constitute a potential risk to public health, these findings underscore the need to control them and to limit bacterial multiplication in bulk tank milk.

Key words: Bulk tank milk- *Echerichia coli- Staph. auerus- L. monocytogenes –Toxic genes*

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1. INTRODUCTION

Milk is an excellent medium for the growth of numerous microbes which produce consequential spoilage of the milk and various milk products or infections in consumers Oliver, et al. (2005). According to the procedure of milk production, it is impossible to avoid contamination of milk with microorganisms therefore the microbial content of milk is a major feature in determining its quality Torkar and Teger (2008). The existence of food borne pathogens in raw milk may increases the threat of ingestion and transmission of food borne pathogens and ingestion of harmful toxins Srinu, et al (2012). Huge numbers of microbes can get access to milk and various milk products including these often listed pathogens in raw milk as *Staphylococcus aureus, Escherichia coli, Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica, Aeromonas hydrophila, Brucella abortus, Campylobacter jejuni, Bacillus cereus, and Listeria monocytogenes* (Garbutt et al., 1997). Enteropathogenic *Escherichia coli* (ETEC) have been implicated in sporadic and epidemic outbreaks of diarrhea in both infants and adults in many parts of the word. ETEC produce one or both of two plasmid-

mediated enterotoxins: a heat-stable enterotoxin (ST) and a heat-labile enterotoxin (LT) Gyles et al., (1974); Smith and Halls (1968) . LT and ST toxin genes are the main pathogenic elements of ETEC strains. These strains are intestinal *E. coli* and cause diarrhea in infected individuals, also can cause urinary hemolytic syndrome which often happens after an intestinal infection Johnson et al., (2002). The most important causes of food borne diseases are shiga toxin producing E.coli (*STEC)* among the other seropathotypes of E.coli. Beutin and Stephan (2006). STEC produce various complications including diarrhea, haemlytic uremic syndrome (HUS) and haemorrhagic colitis (HC) Brett et al., (2003). Report indicate that consumption of raw milk and various milk products related with occurrence of 1 to 5 percent of food infections and among that 53per cent of cases produced by enteropathogenic *E.coli* (EPEC) Schrade and Yager (2001). Humans infected with STEC show symptoms, such as abdominal pain and watery diarrhea, and a number of patients develop a life-threatening disease, such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) Verweyen et al., (2000). The natural reservoirs of STEC are domestic and wild ruminant animals, which shed the bacteria with their feces into the environment Caprioli et al., (2005). STEC-infected animals normally do not show signs of disease and can be included in food production. As a consequence, products of animal origin, such as meat and milk, are at risk of contamination with STEC originating from animals Hussein and Sakuma (2005). Consumption of food containing STEC was identified as a major route of human infections with these pathogens in different countries Caprioli et al (2005); Hussein and Sakuma (2005); Mead et al., (1999). STEC strains can be divided into more than 200 E. coli serotypes. The For STEC, two major types of Shiga toxins, called Stx1 and Stx2, which share 56% homology to each other, have been described previously Paton and Paton (1998). Genetic variants were detected within members of the Stx1 and Stx2 families, and a growing number of toxin types were defined according to differences in toxicity, toxin receptor, and amino acid composition of StxA and StxB subunits Paton and Paton (1998); Scheutz et al., (2001). Some Stx types, such as Stx2 and the elastase (mucus)-activatable Stx2d type, are associated with the high virulence of STEC and with HC and HUS Bielaszewska et al., (2006); Boerlin et al., (1999).

Staphylococcus aureus is one of the most common agents causing food poisoning. It is involved in intramammary infections in bovine causing economic losses and milk safety problems (Taverna et al., 2007). It produce a number of protein, toxins and extracellular virulence factors that one of the most important of them is enterotoxin that cause food poisoning (Orwin et al., 2003). The emetic staphylococcal enterotoxins (SE) are classified as members of the pyrogenic toxin superantigen family because of their biological activities and structural relatedness Dings et al., (2000); Bolaban and Rasooly (2000). Eleven major antigenic types of SEs have been recognised (SEA to SEJ) Monday and Bohach (1999); Tamarapu et al., (2001) and their corresponding genes have been reported Munson et al., (1998). More recently further SE toxins have been identified (SEK, SEL, SEM, SEN, SEO and SEU) Orwin et al., (2001) & Stephan et al., (2001) and the corresponding genes have also been described Letertre et al., (2003) & Omoe et al., (2002). It is known that about 95% of staphylococcal food poisoning outbreaks are caused by SE types SEA to SEE Bergdoll (1983). The remaining 5% of outbreaks may therefore be associated with other newly identified SEs. Staphylococcal enterotoxins are resistant to inactivation by gastrointestinal proteases such as pepsin. Heat resistance is one of their most important physical and chemical properties; their biological activity remains unchanged even after thermal processing of food

(Martin et al., 2004 & Chapaval et al., 2006). For the above mentioned reason, these toxins can cause epidemic gastroenteritis. Actually, SEB is the most important enterotoxin that causes gastroenteritis. The toxins enter from the alimentary tract into the blood circulation. They stimulate the vomiting center of the involuntary nervous system, causing nausea, vomiting, abdominal cramps and diarrhea (Rosec and Gigaud, 2002 & Letertre et al., 2003). Although *Staph. aureus* is not difficult to cultivate and easily identified, there is still need for rapid and sensitive DNA –based assay specific for detecting *S. aureus (*Saei et al., 2010*).*The polymerase chain reaction (PCR), which is a technique for the in vitro amplification of specific segments of DNA, offers a rapid, sensitive and specific identification method for the genes responsible for toxins produced by *Staph. aureus* (Mehrotra et al., 2000 & Anvari et al., 2008). Detection of SE-genes by PCR allows the determination of potentially enterotoxigenic *S. aureus* irrespective of whether the strain produces the toxin or not the inability to detect the enterotoxin by immunological methods may occur due either to low level production of enterotoxin or to mutation in the coding region or in a regulatory region. For this reason, PCR may be considered more sensitive than methods that determine SE-production as immunological methods Zschock et al., (2000) & Holeckova et al., 2002). PCR assays used to identify the pathogen and its enterotoxin genes in food samples can be made in hours rather than days, with high sensitivity and method accuracy, allowing for the detection of very low concentrations of micro-organisms. The PCR assay can detect not only live but also damaged and dead micro-organisms in food subjected to thermal processing Najera-Sanchez et al., (2003*)*.Therefore, there is a need for greater characterization data of such strains from bovine bulk-tank milk because of little data are available in literature for strains in Egypt.

listeria monocytogenes may reach bulk tanks as a result of exogenous contamination via the milking equipment, because of fecal contamination during milking, or, less frequently, by an intramammary route following generalized asymptomatic infection or *mast* Hassan et al., 2001). It is proved that L. monocytogenes grows into biofilms attached to the surfaces in food-processing plants Arizcun, et al., (1998) and Roberts and Wiedmann (2003) and milking systems in dairy farms. The common treatment of surfaces is not effective to eliminate this dangerous foodborne pathogen, and it easily can pass into raw milk. L. monocytogenes can cause a rare but serious disease called listeriosis, especially among pregnant women, the elderly, or individuals with a weakened immune system. L. monocytogenes is more likely to cause death than other bacteria that cause food poisoning. 20 to 30% of foodborne listeriosis infections in high-risk individuals may be fatal Ramaswamy et al., (2007). Detection of *L. monocytogenes* by molecular methods is very specific and can be as fast as the immunological assays Janzten et al., (2006). A number of PCR assays had been described for its detection in foods Levin, (2003). PCR methods had superior sensitivity when compared to standard nucleic acid probes or immunoassays. However, complex sample preparation methods and the use of gel electrophoresis endpoint detection have hampered the transition of these methods from research to routine use in food microbiology laboratories. Nevertheless, factors influencing the performance of conventional PCR in foods continue to be investigated Aznar and Alarcón, (2003). Moreover, recent studies have shown that a broad distribution of identical or closely related enterotoxin-producing *S. aureus* clones is found in bovine mastitis and bulk-tank milk samples (Annemüller et al., 1999; Stephan et al., 2002). Therefore, the objective of the present investigation is (i) to study the occurrence of food poisoning

causing microorganisms (*Escherichia coli, Staphylococcus aureus and Liesteria monocytogens*), (ii) molecular identification of toxigenic genes using polymerase chain reaction (PCR) in isolated strains obtained from bulk tank milk in Gharbia Governorate.

2. MATERIALS AND METHODS

2.1. Samples:

A total of thirty bulk milk samples collected from 3 dairy farms in Gharbia Governorate and subjected to bacteriological examination of food poisoning microorganisms including enumeration of *Staphylococcal aureus* , Coliform count and isolation and identification of *Escherichia coli* and *Liesteria monocytogen,* the obtained isolates were subjected molecular typing of toxigenic genes

2.2. Enumeration of Total Coliform (MPN/g) ICMSF, (1978)

Estimation of coliforms was done by using most probable number technique with MacConkey's broth tubes. A series of 3 fermentation tubes containing MacConkey;s broth and inverted Durham's tubes were inoculated with 1 ml from the previously prepared 10^{th} fold serial dilutions. After thorough mixing, inoculated and control tubes were incubated at 37 °C 24-48 hours. Tubes showing acid and gas were considered as positive for the test. From the laboratory records, the most probable number (MPN) of *coliforms/g.* was calculated by matching with (MPN) table.

Isolation and identification: Samples were processed to isolate the *E. coli* as per the standard *Bacteriological Analytical Manual* (BAM), U.S. Food and Drug Administration (USFDA) method *Kumar et al., (2008).* The samples were enriched in MacConkey broth, and then loopful of culture was inoculated into MacConkey agar. Pink colour colonies obtain from MacConkey agar were taken and inoculate on Eosin methelene blue agar. Greenish metallic sheen colonies obtain on EMB agar were regard as an *E. coli*. Various biochemical tests such as catalase test, Indole production, Methyl red, Voges proskauer, Simon's citrate agar, Urease production, Nitrate reduction etc. were done for the confirmation of *E. coli* as proposed by Edwards and Wing (1972).

2.3. Bacteriological examination of Staphylococcal aureus

- Enumeration of Total Staphylococcal aureus Count: from each dilution 0.1 ml was spread onto a dry surface of double sets of Baird parker agar plate (OxoidCM 275, SR54). Inoculated plates were incubated at 37°C for 48hours. Typical colonies of *S.aureus*(black shining convex colonies, 1-1.5 mm in diameter with narrow white margin and surrounded by a clear zone extending into opaque medium) were enumerated and the average number per gram was calculated APHA, (1992).

2.3.1. Identification

The purified *S. aureus* isolates were identified through different biochemical tests [catalase test, coagulase test (tube test) Quinn, et al., (2002).

2.3.2. Detection of different viulence genes in isolated E.coli and Staph aureus strains by PCR.

DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted

with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in table (1). PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

2.3.3. Analysis of the PCR products.

The products of PCR were separated by electrophoresis on 1-1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

2.3.4. For multiplex PCR, used for toxigenic genes of Staph aureus:

Primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 7 µl of water, and 10 µl of DNA template. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary den.	Sec den	Ann.	Ext.	Final ext.	Reference
stx1	ACACTGGATGATCTCAGTGG	614	94˚C	94˚C	58˚C	72˚C	72˚C	Dipinto *et al.*, 2006
stx2	CTGAATCCCCCTCCATTATG CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	5 min.	1 min	1 min	1 min	10 min	
STa	GAAACAACATGACGGGAGGT GCACAGGCAGGATTACAACA	229		94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.		Lee *et al.*, 2008
Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102		94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.		Mehrotra *et al.*, 2000
Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164						
Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451						
See	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209						
Sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278		94˚C 30 sec.	48˚C 30 sec.	72˚C 30 sec.		

2.4. Bacteriological examination of Listeria monocytogen

One ml of milk sample inculcated in 9ml of *Listeria* enrichment broth (Difco), and incubated at 30°c for 48 hr. After incubation one loopful was subcultured on *Listeria* Oxford medium base. The plates were incubated at 35°c for 24-48 h.

2.4.1. - Identification:

Four typical colonies were transferred from *Listeria* Oxford medium base to Trypticase soy agar with yeast extract for purification. Purified isolates were identified by the Gram- stain, Catalase test, motility test, biochemical tests and Christie- Atkins, Munch- Petersen; test of haemolysis (CAMP Test). Further confirmation of *L. monocytogenes* the isolates were inoculated in to 10% aqueous stock solution of Manitol, L. Rhamnose and D. Xylose FDA (2003).

2.4.2. Polymerase chain reaction (PCR)

*DNA Extraction***:** Boiling method **(**Bansal, 1996). Bacterial pellets were washed once with 1 ml phosphate buffered saline (PBS), pH 7.4, resuspended in a same volume of cold water and incubated in a boiling water bath for 10 min. The clear supernatants obtained after a 5 min centrifugation at 12000g were used for PCR reaction.
Oligonucleotide Primers: in this study 1set of primer was used, *hyl* gene specific for confirmation of *L. monocytogenes* and not any other type of *Listeria*. The sequence, cycling conditions and amplicone size were described in table (2). The PCR products were visualized on 1.3% agarose gel in 1x TBE using GeneRuler 100 bp plus DNA Ladder (Fermentas Cat.No. #SM0323).

Table (2) the sequence, cycling conditions and amplicon size of the used genes:

Gene	Sequence 5\- 3\	Cycling condition	Product size	Reference
Hyl	LM1 CCT-AAG-ACG-CCA-AT C-GAA LM2 CCT-AAG-ACG-CCA-AT C-GAA	Initial denaturation 95ºC for 5 min 30 cycle of 95 ºC for 15 sec 57 ºC for 2 sec 72 ºC for 30 sec Final extention at 72 ºC for 5 min	702	Mengaud et al. (1988)

3. RESULTS

In the present study, table (3) presents the enumeration results for coliform and *Staph. aureus* counts giving an idea about the levels of the concerned pathogens in the 3 dairy farms under investigation. The mean values of total *Coliform counts* for farms I, II and III were $10.5x10^3 \pm 3.1x10^3$, $23.2x10^3 \pm 17.4x10^3$ and $8x10^3 \pm 3.2x10^3$ respectively. The mean values of total *Staph. Count* for farms I, II and III were $45.2x10^3 \pm 6x10^3$, $43.8x10^3 \pm 3.3x10^3$ and $36.3x10^3 \pm 12.2x10^3$ respectively. Also, the presence of food poisoning organisms and isolation rates of *E. coli , S. aurus* and L. monocytogenes have been reported in table (4) in examined BTM samples collected from the three dairy farms (10 samples from each).
*Incidence of E. coli***:** The incidence of *E. coli* was observed in the samples comprising of BTM was (20%), (40%) and (20%), in the concerned dairy farms. Prevalence of *Staph. aureus* was (60%), (40%) and (20%) in the three farms respectively, while L. monocytogenes was

observed in percentage of (10%), (20%) and (10%) respectively. (Table, 4).

Detection of virulence genes of *E.coli*: In this study, the obtained eight E. *coli* identified field isolates by biochemical tests were tested for the presence of *STa* gene. Only one strain obtained from farm I was positive for the presence of *STa gene.* Also, examined for presence of virulence genes (stx1, *stx2)* and none of which were found to be positive.

Detection of enterotoxigenic genes of Staph aureus:

Using multiplex PCR, out of 12 identified field isolates by biochemical tests were tested for the presence of enterotoxigenic gene (*Sea, Seb, Sec, Sed and See).* Two samples were positive to *Sea* gene (16.6 and 25%) one from each farm I and farm II, respectively. The two isolates gave one band at (102 bp) in agarose gel.

All four samples were subjected to PCR from initial culture (Fig. 5) compared to *L. monocytogenes* reference strain, gave a characteristic band at 702 bp to *hyl* gene specific for *L. monocytogenes***.**

Table (3): Statistical analysis of coliform & *staph.aureus* counts in BTM samples in examined farms

	T. coliform Mean±SE	Total staph Mean±SE
Farm I	$10.5x10^3 \pm 3.1x10^3$	$45.2x10^3 \pm 6x10^3$
Farm II	$23.2x10^3 \pm 17.4x10^3$	$43.8x10^3 \pm 3.3x10^3$
Farm III	$8x10^3 \pm 3.2x10^3$	$36.3x10^3 \pm 12.2x10^3$

Table (4): Incidence of food poisoning microorganisms isolated from examined farms.

	Farm I		Farm II		Farm III	
	No.	%	No.	%	No.	%
E. coli	2	20	4	40	2	20
Staph. aureus	6	60	4	40	2	20
Liesteria monocytogenes	1	10	2	20	1	10

Table (5) Incidence of virulence genes in *E. coli* isolates

Farm	*E. coli* isolates	Positive *E.coli* isolates			
		STa		*stx1*	*Stx2*
		No	%		
I	2	1	50	0	0
II	4	0	0	0	0
III	2	0	0	0	0

Table (6) Incidence of enterotoxin genes in *staph aureus* isolates

Farm	No of *S. aureus*	Positive *S. aureus* isolates for presence of enteric genes					
		Sea		*Seb*	*Sec*	*Sed*	*See*
		No	%				
I	6	1	16.6	0	0	0	0
II	4	1	25	0	0	0	0
III	2	0	0	0	0	0	0

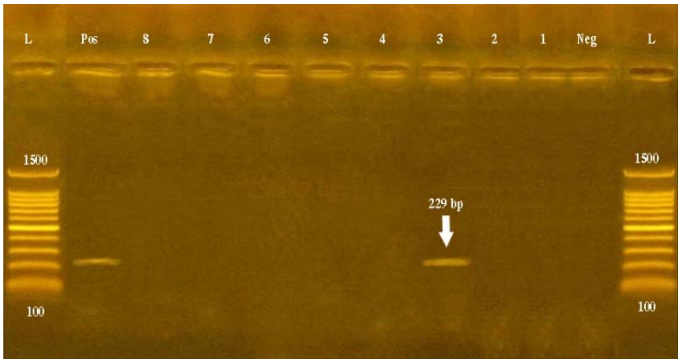

Figure-1. Agarose gel showing PCR amplification of *E. coli STa* gene product (229 bp) Pos Positive control, Neg: Negative control, L: DNA Ladder, Lane 1: positive E. coli strains and Lane 2 to 8 : negative E. Coli strains

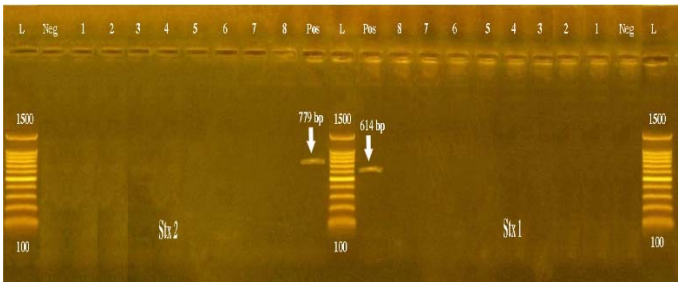

Figure-2. Agarose gel showing PCR amplification of *E. coli stx1, stx2* gene product (614, 779 bp) Pos: Positive control, Neg: Negative control, L: DNA Ladder, Lane 1 to 8: *E. coli* isolates

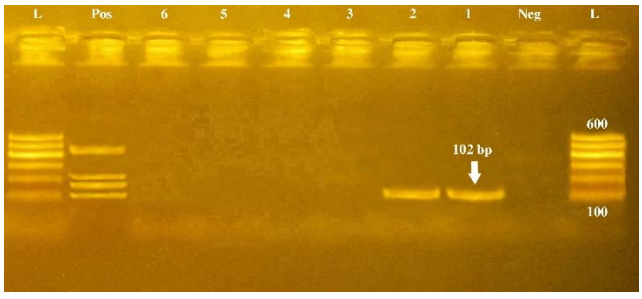

Figure -3 Agarose gel electrophoresis showing the results of multiplex PCR for detection of enterotoxin genes among the *S. aureus* isolates. Lane L: 100 bp ladder DNA molecular weight marker (Qiagen), Lane Pos: positive control for *Sea, Seb, Sec* and *See* genes, Lane Neg : negative control, Lane 1: positive *Sea S. aureus* isolated from farm I BTM sample, Lane 2: positive *Sea S. aureus* isolated from farm II BTM sample, Lanes 3 to 6 : no amplification.

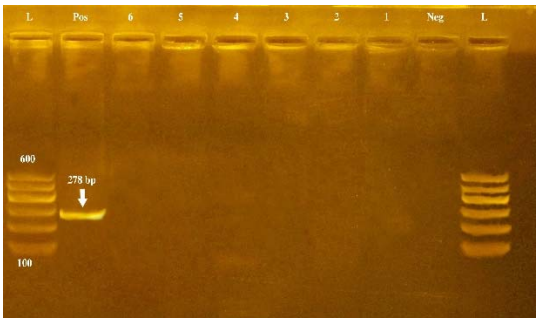

Figure-4. Agarose gel showing monoplex PCR amplification for detection of enterotoxin gene *sed* among the *S. aureus* isolates. Lane L: 100 bp ladder DNA molecular weight marker (Qiagen), Lane Pos: positive control for *Sed* gene (278 bp), Lane Neg : negative control, Lane 1 to 6: negative *S. aureus* isolates obtained from farm I BTM and farm II BTM samples for *Sed* gene : no amplification.

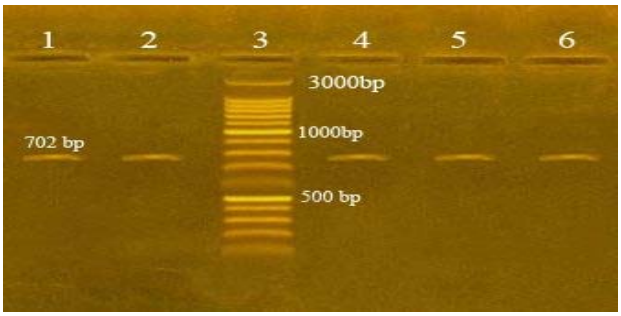

Figure-5: Agarose gel electrophoresis of the amplification products of L. monocytogenes DNA obtained from tested BTM samples strains compared to Reference strain using *hyl* gene . Lane 1 - *L. monocytogenes* Reference strain (702bp), Lane 2: farm I positive band, Lane 3: Marker (Fermentas), Lane 4, 5: farm II positive bands, Lane 6: farm III positive band

4. Discussion

The safety of milk is an important attribute of consumers of milk and dairy products. Milk pasteurization safeguards consumers from many potential food borne hazards in milk and milk products. Despite the pasteurization process, the quality and safety of raw milk are important in reducing the risk of food borne diseases associated with milk because raw milk is the starting point of the milk production-consumption chain. The presence of food poisoning organisms in raw milk generally comes from cows with mastitis, handlers or deficient hygiene. When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections *(Quintana and Carneiro, 2006).* In the present study total of 30 BTM samples 10 of each were collected from 3 dairy shops in Gharbia Governorate, Egypt. These samples were investigated bacteriologically to detect occurrence of *E.coli, S. aureus* and *L. monocytogenes* among the examined samples.

Table (3) illustrates the mean values of total *Coliform counts* for farms I, II and III were $10.5x10^3 \pm 3.1x10^3$, $23.2x10^3 \pm 17.4x10^3$ and $8x10^3 \pm 3.2x10^3$ respectively, but this results came in contrast of results reported by *Sobih, (2000)* and *Gillespie et al., (2012)* who found higher findings of Coliform count . The current results were lower than those reported by *Gihan, (1997)* and *Jayarao and Wang, (1999)* but they were nearly similar to results reported by *Hassan and Al-Sanjary, (1999).* Colifrom counts of raw bulk tank milk should be routinely performed to identify bacteria that

originate from fecal contamination of milk. Colifrom bacteria can contaminate milk through poor udder preparation or unhygienic handling of the milking machines.

The mean values of total *Staph.* Count for farms I, II and III were $45.2x10^3 \pm 6x10^3$, $43.8x10^3 \pm 3.3x10^3$ and $36.3x10^3 \pm 12.2x10^3$, respectively, observed in Table (3). Nearly similar results were obtained by *Gillespie et al., (2012).* Staph. aureus is one of the causative agents of mastitis in dairy herds (*Barkema et al., 2006).* This disease involves inflammation of the mammary glands and a resultant sporadic shedding of Staph. aureus cells into the raw milk (*Barkema et al., 2006*). Therefore, the presence of large concentrations of Staph. aureus is indicative of mastitis in a dairy herd. From a food safety perspective, it is recognized that Staph. aureus is an enterotoxin-producing pathogen but that the concentration needs to exceed 10^5 cfu/ml for sufficient toxin to be produced to cause human illness (Hill, 1981; Jay, 2000). None of the raw milk samples in this study contained numbers of S. aureus that were close to this count

Results in Table (4) showed the incidence of E. coli was observed in the samples comprising of BTM was (20%), (40%) and (20%), higher incidence of E. coli (52%) was observed in Virpari et al., (2013) and in Soomro et al., (2002) was 57%, while nearly similar results (26.4%) was reported by Bandyopadhyay et al., (2011) and (30.2%) by Farzan et al., (2012). Incidence of Staph. aureus was (60), (40) and (20) % in the three farms respectively. Similar results of *Staphylococcus* species isolation was observed in raw milk samples (56%) reported by EL-Jakee et al., (2013) and Stephan et al., (2001) showed only 32.4% *Staph. aureus* and Khudor et al. (2012) where *S. aureus* isolated from raw milk by percentage of 28.5% . Lower results of raw milk were observed with that of Rahimi and Alian (2013) as they isolate *Staph. aureus* from raw milk by percentage of 17.5%.on the other hand higher results were reported by Rall et al.(2008) isolated *Staph. aureus* from raw milk by percentage of 68% and 70.4% respectively.

In the current study the isolated *L. monocytogenes* found in percentage of (10%), (20%) and (10%) respectively. Lower incidence of observed as 5.1% in raw milk samples (*Kalorey et al., 2008).* The source of *L. monocytogenes* in raw milk is mostly the gastrointestinal tract of animals and the environment, skin of the teats, in particular shedding of *Listeria* into milk due to chronic mastitis (O'Donnell, (1995) is less frequent. Waak et al. (2002) studied the incidence of Listeria species in raw whole milk from farm bulk tanks and from raw milk and L.monocytogenes was found in 1.0 % of 294 farm bulk tank milk.

LT and ST toxin genes are the main pathogenic elements of ETEC strains. These strains are intestinal E. coli and cause diarrhea in infected individuals ,also can cause urinary hemolytic syndrome which often happens after an intestinal infection Johnson et al., (2002). In this study, results observed in table (5) revealed that, the obtained eight *E. coli* identified field isolates by biochemical tests were tested for the presence of *STa* gene. Only one strain obtained from farm I was positive for the presence of *STa gene.* Nearly similar results observed in *Jung, (1999)* identified 3 strains of E.coli containing only the STa gene and only one strain containing LT and STa . Also, the obtained eight E.coli strains examined for presence of virulence genes (*stx1, stx2)* and none of which were found to be positive

Shiga toxin-producing E. coli are highly pathogenic in humans with low infectious doses Hussein and Sakuma, (2005). Among the STEC, O157:H7 is the classical serotype associated with enterohemorrhagic diseases. Non- O157 STEC strains are only recently becoming recognized as important human pathogens (Nataro and Kaper, 1998; Hussein and Sakuma, 2005). Consumption of raw milk has been implicated as the cause in several outbreaks of disease caused by *E. coli* O157:H7 and by non-O157 STEC

(Hussein and Sakuma, 2005). Shiga toxin-producing *E. coli* excrete potent Shiga toxins that are encoded by the *stx*1 and *stx*2 genes, respectively (Hussein and Sakuma, 2005). The STEC isolates in this study predominantly carried the *stx*2 gene. Epidemiological data suggest that *stx*2 is more important than *stx*1 in the development of hemolytic uremic syndrome, a life-threatening illness associated with STEC infection in children (Nataro and Kaper, 1998). Results in table (6) revealed that the suspected STEC isolates none of them harboring the *sx1and sx*2. On the contrary, a study conducted by Steele et al. (1997) reported that only 0.87% of the BTM samples in Ontario contained STEC. Four of the five isolates of E. coli encoded for shiga-toxin 2 gene while one strain encoded for shiga-toxin 1 gene. Also, Montenegro et al. (1990) reported that most of the STEC isolates of bovine origin encoded for shiga-toxin 1 gene. Virpari et al., (2013) reported that out of 80 *E. coli* isolates, 12 (15.00%) *E. coli* isolates found positive for *stx*1gene and 18 (22.50%) *E. coli* isolates found positive for *stx*2 gene. Similar finding of predominance of *stx*2 producing strains were reported by Sabry and Elmalt 2008)

Milk is a good substrate for S. aureus growth and for enterotoxin production. In addition, enterotoxins retain positive their biological activity even after pasteurization Asao et al., (2003).The determination of staphylococcal enterotoxin type has a long history of successful use in epidemiological studies in both clinical and environmental microbiology studies. Oligonucleotide primers for specific detection of enterotoxin genes sea, seb, sec, sed, and see have previously been reported (Johnson et al.,1991& Tsen et al., 1994), these were used in individual PCR assays, thus requiring several PCRs for each sample to screen for the presence of all of the enterotoxin genes. Monday and Bohach (1999) have recently described a multiplex PCR assay for the detection of all of the *staphylococcal* enterotoxin genes, but again this assay uses separate primer pairs for each toxin gene to be detected. Generally, five classical *staphylococci* enterotoxin (SE) SEA to SEE were recognized. It was shown that about 95% of staphylococcal foodpoisoning outbreaks were caused by strains carrying the classical SE and the remaining 5% of coagulase positive species; *S. hyicus* and *S. intermedius* outbreaks were associated with other identified (Wang et al., 2012). Using multiplex PCR, out of 12 *S. aureus* isolated from the examined samples, 1 (16.6%) and 1(50%) isolates were positive for sea could produce enterotoxins as shown in table (6) isolated from farm I & II respectively. Others found sea but with different percentage than our study as Adwan et al.(2005); Rall et al.(2008); Rahimi et al. (2012) & ElJakee et al.(2013); 7.1%,41%, 12.7% and 40% respectively. While Veronica et al.(2011) & Khudor et al.(2012) didn't find sea at all. None of the 12 isolates harboring other *SEs* genes. For sec results, our results agree with Rahimi and Alian (2013) didn't find sec at all. While Sharma et al.(2000)& Khudor et al.(2012) found only sec by percentage of 11.1%, 19% and 18.5% respectively and none of these isolates harboring other SEs genes. The current study, there is no gene in genes coding for more than one enterotoxin in one sample while Veronica et al.(2011) found a combination between sea-sed-see by percentage of 1.1% and El-Jakee et al.(2013) found combination between seb-sed by percentage of 20%. On the other hand, enterotoxin A was the most commonly produced toxin. Moreover, enterotoxin A is most often implicated in cases of staphylococcal food poisoning (Shale et al., 2005). The dominance of *S. aureus* enterotoxin A isolates in our present study has been also reported by other researchers for *Staph. aureus* recovered from food samples (Tsen et al., 1998 & Bendahou et al., 2009). The SEs could be able to indicate the origin of the *Staph.aureus* strains because it was observed that a higher ratio of isolates from

bovine produced SEC and those from human produced mainly SEA (Ahari et al., 2009). The ability of *Staph. aureus* isolates to produce one or more SEs in food products is linked to *staphylococcal* food poisoning (Bennett, 2005). Enterotoxigenic strains of *Staph. aureus* have been reported to cause a number of diseases or food poisoning outbreaks in many countries because of ingestion of contaminated dairy products or milk with staphylococcal enterotoxins (Oliver et al., 2005; Ikeda et al., 2005 & ISfID, 2010). In the present work 83.3% of *Staph. aureus* isolates were negative to the five classical enterotoxin genes.This might be explained by the fact that these isolates either have not harboured any gene of enterotoxins or thy might have other types of SEs which are family of 18 serological types of heat stable enterotoxin (MacLauchlin et al., 2000; Ikeda et al., 2005; Rall et al., 2008 & Bhunia, 2008). Differences in SE type prevalence compared with the present study likely reflect the distinct origin of the isolates. It can be considered that food handlers are the most usual contamination source leading to food poisoning. Nevertheless, since these toxins resist heat treatment, the present findings indicate a potential public health hazard and underscore the need to establish both effective bovine mastitis control programs and proper milk cooling methods to limit *Staph. aureus* presence and multiplication in bulk tank milk. (Veronica etal., 2011). Rapid isolation and confirmation methods for *L. monocytogenes* in foods are still being sought *(Beumer and Hazeleger, 2003).* But there are certain strains of *L. monocytogenes* which behave phenotypically quite typical and inconspicuous but are non-pathogenic (Hof and Rocourt, 1992). Six Listeria species are known to exist an attempt to identify *L. monocytogenes* by PCR-based detection, potentially suitable oligonucleotide primer sequences complementary to the virulence gene hlyA (GenBank accession no. AF253320) and known to be specific for *L. monocytogenes*.

Thus, it has been suggested that diagnosis of pathogenic Listeria spp. And listeric infection should ideally be based on virulence markers (Notermans et al., 1991). Moreover, the importance of PCR has been investigated for detection of L. monocytogenes from foods (Gouws and Liedemann, 2005). Thus, in the present study *L. monocytogenes* strains isolated from BTM samples were analyzed for the presence of virulence-associated gene *hlyA* of L. monocytogenes employing the monoplex PCR for rapid comparing and confirmation with conventional culture method. All four samples were subjected to PCR from initial culture (Fig. 5) compared to *L. monocytogenes* reference strain, gave a characteristic band at 702 bp to *hylA* gene specific for *L. monocytogenes* so the results obtained from isolation were in line with that of molecular diagnosis (PCR), while (Gouws and Liedemann, 2005) reported that only 37 % of samples were confirmed to be positive for *L. monocytogenes* by PCR amplification of the *hly* gene (732 bp). PCR was able to eliminate the false positives and detect all *L. monocytogenes* in the food products, unlike the conventional methods used in the industry. In addition to the fact that the incidence of *Listeria* species was higher than *L. monocytogenes* on selective media, there was also the presence of *Listeria*-like organisms. These organisms had the typical appearance of *Listeria* on selective media, but were non-*Listeria* species. PCR proves to be a sensitive and rapid technique to be included in the procedure of detection of *L. monocytogenes* in food products.

Dairy products contaminated with *Listeria monocytogenes* have been responsible for human listeriosis outbreaks Dalton et al., (1997). The serious consequences of listeriosis, such as a septicemic form of the illness in elderly and immune compromised people, and abortion in pregnant women or death of their newborn, constitute a serious threat to public health Even though the

symptoms may be relatively mild in the mother, the illness may be transferred to the fetus causing serious illness or fetal death. Some symptoms of Listeriosis may include meningitis, encephalitis, septicemia, spontaneous abortion, still birth, and influenza-like symptoms (Sutherland et al., 2003; Azevedo et al., 2005). Antibiotic treatment of pregnant women or immunocompromised people who have eaten food contaminated by *L.monocytogenesis* can prevent the most serious consequences of listeriosis, but only if the infection is diagnosed in time. Another complication is that *Listeria* is able to grow well at law temperatures. Thus, refrigeration is not as effective in preventing growth of *Listeria* in food as it is for most other bacteria that cause food-born disease (Salyers and Whitt 2002)

Conclusions: Current study supports the finding that BTM can be regarded as critical source of pathogenic *E. coli, Staph. aureus and L. monocytogenes* This explains the need of strict monitoring and surveillance for effective measures of hygiene and sanitary practice during production of milk in farms.

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التصنيف الجزيئي للميكروبات المعدية المسببه للتسمم الغذائي في الالبان المجمعه في محافظه الغربيه

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الملخص العربي

اجريت هذه الدراسة لتحديد مدي انتشار ميكروبات التسمم الغذائي في خزانات الحليب في ثلاث مزارع بمحافظه الغربيه وتم الكشف عن تواجد كل من ميكروب الايشريشيا كولاي، المكور العنقودي الذهبي والليستريا مونوسيتوجين في عينات خزانات الحليب في المزرعه الاولي بالنسب الاتيه 20و60و40 على التوالي، وفي المزرعة الثانية بالنسب الأتية 40و 40و20 على التوالي والمزرعة الثالثة بالنسب الأتية 20و20و10 على التوالي. يعتبر تفاعل البلمره المتسلسل تقنيه قويه مؤكده للكشف عن مسببات الامراض في الاطعمه كما انه يتميز بالخصوصيه والحساسيه والكشف السريع في التعرف علي الميكروبات من مصادر مختلفه لذا استهدفت هذه الدراسه الكشف عن جينات السميه في معزولات الميكروبات الثلاث لبيان مدي ارتباط هذه المعزولات بحالات التسمم الغذائي الناتجه من استهلاك منتجات الالبان المصنعه من تلك الالبان بالكشف عن جينات الزيفانات المعويه المقاومه للحراره STa وجينات الضراوه المسئوله عن افراز السموم المشابه لسموم ميكروب الشيجلا sx1, sx2 في معزولات الايشريشيا كولاي الثمانيه وقد وجدت عتره واحده ايجابيه لوجود الجين المسئول عن افراز السموم المقاومه للحراره في المزرعه الاولي ولم تتواجد جينات sx1, sx2 في اي من المعزولات لالثمانيه بافراز تلك السموم . كما تم الكشف عن الجينات المسئولة افراز السموم المعوية في ميكروب المكور العنقودي الذهبي لانتاج السموم المعويه Ses. كان الكشف باستخدام تفاعل البلمرة المتعدد ومن أصل 12 معزولة لميكروب اللمكور العنقودي الذهبي من عينات المزارع الثلاث كانت عتره واحده (16,6 %) من المزرعة الاولي وعتره ثانيه من المزرعه الثانيه (25%) تحمل الجين. كما استخدم اختبار البلمره المتسلسل في التشخيص الجزيئي لميكروب اليستريا مونوسيتوجين بالكشف عن جين لتأكيد التعرف على الليستريا مونوسيتوجين وقد تم تاكيد الاربع عترات المعزوله من المزارع لهذا الجين. hyl وبم ان تواجد هذه الميكروبات المسئوله عن التسمم الغذائي في تنكات البان المزارع تشكل خطرا محتملا علي الصحه العامه فان هذه النتائج تؤكد الحاجه الي السيطره عليها والحد من تكاثر البكتريا في تنكات الحليب المجمعة.

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