Occurrence and molecular characterization of some zoonotic bacteria in bovine milk, milking equipments and humans in dairy farms, Sharkia, Egypt

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Abstract

One hundred bulk tank milk samples, 50 milking equipments' swabs and 50 dairy workers' hand swabs were collected from three dairy farms at Sharkia Province, Egypt, to determine the occurrence of some zoonotic bacteria as well as to investigate the presence of some virulence genes in all isolates by PCR. *Staph. aureus*, *St. agalactiae*, *Salmonella* species and *M. bovis* were isolated from milk samples with the percentages of 17%, 4%, 9% and 1%, respectively. In dairy workers hand swabs, the respective isolation rates of *Staph. aureus*, *St. agalactia* and *Salmonellae* were 10%, 2% and 8%, while only *Staph. aureus* and *St. agalactiae* were recovered from milking equipments (6%, each). Of the examined *Staph. aureus* isolates, 11.8% and 20% of milk and hand swabs' isolates, respectively, were carrying *sea* gene. In *St. agalactiae* isolates, *cfb* virulence gene was identified in all the obtained isolates. Moreover, *spvC* virulence gene was detected in 40% and 33.3% of *S.* Typhimurium isolates recovered from milk and hand swabs' samples, respectively. While the respective percentages of *spvC* gene identification in *S.* Enteritidis isolates from the same sources were 75% and 100%. *M. bovis* was isolated from 1% of milk samples only. In conclusion, the isolation of potentially virulent *Staph. aureus*, *St. agalactia* and *Salmonellae* in dairy farms is of public health significance to milk consumers. Although the low isolation rate, the detection of *M. bovis* in milk samples should be of great concern due to its high public health hazards.

Introduction

Milk is considered one of the high nutritional quality foods; however, it is an excellent medium for growth and transmission of different bacterial pathogens to humans (Donkor *et al.*, 2007). Microbial contamination of milk can occur from a variety of sources such as cows with mastitis, milk handlers, milking equipments or deficient hygiene (Bramley and Mckinnon, 1990; De Oliveira *et al.*, 2011). There is an increasing concern about the presence of zoonotic pathogens in raw milk due to the spread of such pathogens between cows during the milking time by milking equipments and milkers' hands (Fox and Gay, 1993). Therefore it is essential to assess the role of raw milk and other possible sources of contamination within the farm in transmitting some zoonotic bacteria to milk consumers.

Enterotoxigenic *Staphylococcus aureus* is considered the world's third most important cause of foodborne intoxication (Tirado and Schmidt, 2001). Eleven antigenic types of *Staph. aureus* enterotoxins (SEs) have been recognized and they retain their biological activity even after pasteurization (Asao *et al.*, 2003). Thus, because of the importance of SEs in

the public health and food sector, efficient screening to detect their prevalence is required (Morandi *et al.*, 2007). *Streptococcus agalactiae* or group B *Streptococcus* (GBS) is associated with early and late onset disease in infants, with asymptomatic colonization of the uro-genital and gastro-intestinal tract (Zadoks *et al.*, 2011). It is also implicated in causing septicemia and other clinical manifestations in non-pregnant adults. Moreover, *St. agalactiae* has been widely reported as an important cause of mastitis in cattle (Amosun *et al.*, 2010).

Non-typhoid *Salmonella* species are considered the most important bacterial etiology for enteric infections worldwide including Egypt (Bulgin *et al.*, 1982). Different serotypes of *S. enterica* have been isolated from dairy animals and their environment, some of which are considered pathogenic to humans (Blau *et al.*, 2005). Human infection is mostly associated with consumption of food of animal origin including milk (Gomez *et al.*, 1997).

Bovine tuberculosis caused by *Mycobacterium bovis* is a relevant zoonosis that can spread to humans through inhalation and consumption of milk (Thoen and Barletta, 2005). *M. bovis* is classified as Risk 3 pathogen for public health (OIE, 2005) and it has

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been associated with extra-pulmonary tuberculosis in infants and children due to ingestion of milk (Thoen *et al.*, 2006). The objective of the current study was to investigate bacterial contamination of milk, milking equipments and milker's hands in dairy farms with some zoonotic bacteria and to molecularly characterize the potential of some isolates to harbor virulence genes.

Material and Methods

Sampling

A total of 100 bulk tank milk samples of cattle origin, 50 swabs from milking equipments and 50 hand swabs from dairy workers were collected from three randomly chosen dairy farms in Sharkia, Egypt. The farms included two small (up to 25 animals) and one medium sized (up to 50 animals) farms at Azizia (Menia Elkamh), Belbeis and Bani-Helal (Menia Elkamh), respectively. The farms used automatic milking except the small sized farm in Belbeis used manual milking. The produced milk in Azizia and Bani-Helal farms is distributed to milk factories and dairy products outlets, while the milk produced in Belbeis farm is distributed to street vendors. The study was conducted during the period from February to June, 2013.

Milk samples

Bulk tank milk samples were collected aseptically into screw capped bottles and kept at 4ºC until microbiological examination. Twenty five ml from each sample were homogenized with 225 ml of buffered peptone water (BPW) (Oxoid, CM509, Adelaide, Australia) for pre-enrichment and incubated at 37ºC for 24 h (Addis *et al.*, 2011a).

Hand and milking equipments' swabs

Moistened sterile swabs were rolled over the palm of hands, area between fingers, finger tips and nails. Also, swabs from milking equipments were collected. Each swab was inserted in tubes containing BPW for pre-enrichment.

Isolation and identification of Staph. aureus

A loopful from the pre-enriched culture homogenate in BPW was inoculated onto Baired parker agar medium (Oxoid, CM0275, Adelaide, Australia) (Iso-6888-1, 1999) and incubated at 37ºC for 24 hours. The suspected colonies were subjected to Gram staining and biochemical identification using catalase test, coagulase test (test tube method) and oxidase test (Cruickshank *et al.*, 1975).

Isolation and identification of St. agalactiae

The pre-enriched culture homogenate in BPW was inoculated onto Edward's media plates (Oxoid CM0027, Adelaide, Australia) and incubated at 37ºC for 24-72 hours. Suspected colonies were subjected to Gram staining and biochemical identification using catalase, oxidase, esculin hydrolysis, CAMP test and carbohydrates utilization (Cruickshank *et al.*, 1975). The biochemically identified *St. agalactiae* strains were serogrouped with a commercial latex agglutination kit (Oxoid, Adelaide, Australia) for the identification of streptococcal groups A, B, C, D, F and G.

Isolation and identification of Salmonellae

The isolation and identification of *Salmonellae* were done according to ISO 6579 method (Iso-6579, 2002; 2007). Pre-enriched culture homogenate in BPW were enriched in Rapapport Vassilidis (RV) broth (Oxoid, CM669, Adelaide, Australia) and incubated at 41ºC, moreover, another portion of the enriched broth was enriched in Muller–Kauffmann tetrathionate/ novobiocin broth (Oxoid, CM1048, Adelaide, Australia) and incubated at 37ºC for 24 hs ± 2 hs. A loopful from the enriched broth was then streaked onto the surface of Xylose Lysine Deoxycholate (XLD) agar plates (Oxoid, CM469, Adelaide, Australia) and Brillient Green agar plates (Oxoid, CM0263, Adelaide, Australia) and incubated at 37ºC for 24 hours. The suspected *Salmonella* colonies were subjected to Gram staining and biochemical identification using oxidase test, hydrolysis of urea, H_2S production and lysine decarboxylation (Cruickshank *et al.*, 1975). The biochemically identified *Salmonella* isolates were then subjected to serotyping following Kauffman-White Scheme with commercial antisera (Difco Laboratories Deteroeit, Mitchigeu, USA) for cell wall (O) and flagellar (H) antigen identification (Kauffman, 1974). Serological identification was carried out at Animal Health Research Institute, Dokki, Giza.

Isolation of M. bovis

Milking equipments' swabs and hand swabs pre-enriched in BPW were streaked directly onto Löwenstein-Jensen media (Oxoid CM0884, Adelaide, Australia). While, for each milk sample, 8 ml were centrifuged at 10,000 rpm for 20 minutes and the supernatant was discarded. Decontamination of the fat and protein sediment was then performed by Petroff decontamination method by 4% sodium hydroxide and 4% sulfuric acid (Balian *et al.*, 2002) and then a loopful was streaked onto Löwenstein-Jensen media.

The samples were incubated at 37°C for 90 days with weekly examination. The isolation of *M. bovis* was carried out in a class II biological safety Type A2 cabinet (NewTechnology) that has been cleaned with 10% sodium hypochlorite and 70% alcohol, followed by exposure to ultraviolet light for 30 minutes with air circulation system turned on (Franco *et al.*, 2013).

DNA extraction

DNA extraction from biochemically identified *Staph. aureus*, *St. agalactiae*, and *Salmonella* isolates was performed using Bacterial DNA extraction kit (Spin-Column) (BioTeke Corporation, Shanghai, China) according to the manufacturers guidelines. The DNA from suspected *M. bovis* cultures was extracted using GF-1 Tissue DNA Extraction Kit according to the manufacturer guides (Vivantis Technology, GF-TD-050).

Multiplex PCR for identification of Staph. aureus enterotoxins

Purified DNA of *Staph. aureus* isolates was subjected to a multiplex PCR for the identification of enterotoxins (Cremonesi *et al.*, 2005). Oligonucleotide primers sequences (AlphaDNA, Montreal, Quebec, Canada) for the amplification of thermostable nuclease gene (*nuc*), enterotoxins *sea*, *sec* and *sed* genes are shown in Table 1. The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2x power Taq PCR mastermix (BioTeke Corporation, Shanghai, China) and 20 µM each *sea*, *sec* and *sed* primers, 30 µM each *nuc* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA was run in the PCR as a negative control.

The reaction conditions consisted of one cycle of 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 68°C for 1 min and a final cycle of 72°C for 7 min. The reaction was carried out in Primus Thermal Cycler (MWG-Biotech Thermal Cycler, Ebersberg, Germany). Amplification products were resolved in 1.2% (w/v) agarose gels along with 100 bp molecular weight ladder (BioTeke Corporation, Shanghai, China). The agarose gel was prepared in 1 x TBE (89 mM Tris- Borate, 2 mM EDTA, and pH 8.3) stained with 5 µM ethidium bromide. The gels were run in 1xTBE, 5 µM ethidium bromide for at least 45 minutes at 100 volts and then visualized under Ultra Violet light of ultraviolet transilluminator (Spectroline, Westbury, NY, USA).

Molecular identification of St. agalactiae virulence gene

Purified DNA of *St. agalactiae* isolates was subjected to PCR for the screening of CAMP virulence factor specific gene (*cfb*) (Chen *et al.*, 2005). The oligonucleotide primers sequences (AlphaDNA, Montreal, Quebec, Canada) are listed in Table 1. The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2x power Taq PCR mastermix (BioTeke Corporation, Shanghai, China), 0.5 µM each *cfb* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA was run in the PCR as a negative control. The reaction conditions consisted of one cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec and a final cycle of 72°C for 5 min. The gel preparation, electrophoresis and visualization were performed as previously mentioned in *Staph. aureus* section.

Molecular identification of Salmonella species virulence gene

Purified DNA of *S.* Typhimurium and *S.* Enteritidis isolates was subjected to PCR for the identification of *Salmonella* virulence plasmid (*spvC*) (Betancor *et al.*, 2010). The oligonucleotide primers sequences (AlphaDNA, Montreal, Quebec, Canada) are listed in Table 1. The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2x power Taq PCR mastermix (BioTeke Corporation, Shanghai, China), 0.6 µM each *spvC* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA was run in the PCR as a negative control. The reaction conditions consisted of one cycle of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 51°C for 45 sec and 72°C for 1 min, and a final extension at 72°C for 10 min. The gel preparation, electrophoresis and visualization were performed as previously mentioned in *Staph. aureua* section.

Real time PCR for identification of M. bovis

Extracted DNA from the suspected *M. bovis* colonies were identified by performing a real time PCR using MTplex dtec-RT-qPCR Test (Edifici-Quórum3, Spain) that comprises a series of species-specific targeted reagents designed for detection of all species contained in the *Mycobacterium tuberculosis* complex.

The primers and TaqMan probe target a sequence conserved for all strains of each single species belonging to *Mycobacterium tuberculosis* complex. According to the source of *Mycobacteria* isolation, the species is determined, in the current study, the source of isolation was milk of bovine origin; therefore, the positive reaction using MTpleax reaction refers to contamination with *M. bovis*. Moreover, a screening test for tuberculosis was carried out for all dairy

Table 1. Primer sequences and expected product sizes

Organism	Gene	Primer sequence (5'→3')	Expected product size (bp)	Reference
Staph. aureus	nuc	NUC-F166 AGT TCA GCA AAT GCA TCA CA NUC-R565 TAG CCA AGC CTT GAC GAA CT	400 bp	(Cremonesi et al., 2005)
	sea	SEA-F1170 TAA GGA GGT GGT GCC TAT GG SEA-R1349 CAT CGA AAC CAG CCA AAG TT	180 bp	
	sec	SEC-F97 ACC AGA CCC TAT GCC AGA TG SEC-R467 TCC CAT TAT CAA AGT GGT TTC C	371 bp	
	sed	SED-F578 TCA ATT CAA AAG AAA TGG CTC A SED-R916 TTT TTC CGC GCT GTA TTT TT	339 bp	
St. agalactiae	cfb	cfb-forward: GCTGTTTGAAGTGCTGCTTG cfb-reverse: GACTTCATTGCGTGCCAAC	288 bp	(Shome et al., 2012)
Salmonella	spvC	spvC-forward: CCCATAAATAGGCCTAATCT spvC-reverse: TTACTCTGTCATCAAACGAT	700 bp	(Haneda et al., 2001)

Table 2. Prevalence of some zoonotic bacteria in milk, milking equipments and milkers' hands

Type of samples	Staph. aureus	St. agalactiae	Salmonella species			M. bovis
			Overall	S. Enteritidis**	S. Typhimurium**	
Bulk tank milk (n=100)	17 (17%)*	4 (4%)	9 (9%)	5 (55.6%)	4 (44.4%)	1 (1%)
Milking equipments (n=50)	3 (6%)	0	3 (6%)	2 (66.7%)	1 (33.3%)	0
Milkers' hands (n=50)	5 (10%)	1 (2%)	4 (8%)	3 (75%)	1 (25%)	0

*The percentage of isolation is indicated between brackets
**The percentage of Salmonella serotypes was calculated in relevance to the overall Salmonella species isolates number

Table 3. Prevalence of virulence genes in Staph. aureus, St. agalactiae and Salmonella isolates

Sources of bacterial isolates	Identified virulence genes						
	Staph. aureus genes				St. agalactiae cfb	Salmonella spvC	
	nuc gene	sea	sec	sed		S. Enteritidis	S. Typhimurium
Bulk tank milk	2/17 (11.8%)*	2/17 (11.8%)	-	-	4/4 (100%)	2/5 (40%)	3/4 (75%)
Milking equipments	-/3	-/3	-	-	-/-	-/2	-/1
Milkers' hands	1/5 (20%)	1/5 (20%)	-	-	1/1 (100%)	1/3 (33.3%)	1/1 (100%)
Total	3/25 (12%)	3/25 (12%)	-	-	5/5 (100%)	3/10 (30%)	4/6 (66.7%)

*The percentage of isolation is indicated between brackets

workers to ensure that they are not the source of contamination of milk. The results of the screening test were negative (data not shown).

The reaction of 20 µl final volume consisted of 10 µl Hot Start-Mix qPCR 2x, 1 µl MTplex dtec-qPCR-mix, 4 µl DNase/RNase free water and 5 µl DNA sample. The suspected sample was diluted in duplicate of two fold dilution. Five reaction mixtures with no added DNA were run in the same reaction as negative controls. Another reaction mixture with an undiluted positive control (Standard Template MTplex positive control) and a duplicate of two fold dilutions of the positive control were also run.

The reaction conditions consisted of one cycle of 95°C for 5 min followed by 45 cycles of 95°C for 30 sec and 60°C for 60 sec for hybridization, extension and data collection. The reaction was run in Applied Biosystem StepOne Real Time PCR System and FAM fluorogenic signal was collected and the cycle threshold of the reactions was detected by StepOne™ software version 2.2.2 (Life Technology).

Results

Prevalence of some zoonotic bacteria in milk, milking equipments and milkers' hands

One hundred bulk tank milk samples, 50 milking equipments' swabs and 50 hand swabs from dairy workers were examined for the prevalence of Staph. aureus, S. agalactiae, and Salmonella spp. by cultural, biochemical and serological characterization. The results show that the prevalence rates of the aforementioned bacteria in the examined milk

samples were 17%, 4% and 9%, respectively (Table 2). The respective prevalence rates from milking equipments' swabs were 10%, 2% and 8%. Only Staph. aureus and Salmonella spp. were isolated from 6% of milk and hand swabs, each.

Only S. Enteritidis and S. Typhimurium were identified during the current study. The respective prevalence rates of S. Enteritidis and S. Typhimurium from milk were 55.6% and 44.4%, from milking equipments were 66.7% and 33.3%, while in hand swabs were 75% and 25% (Table 2). M. bovis prevalence was determined by cultural characterization and real time PCR from 1% of milk samples only (Table 2).

Prevalence of some virulence genes in Staph. aureus, St. agalactiae and Salmonella isolates

Staph. aureus isolates from 11.8% of milk and 20% of hand swabs were shown to harbor sea gene responsible for the production of enterotoxin A (Table 3 and Figure 1). All St. agalactiae isolates from milk and hand swabs were positive for cfb gene (Table 3 and Figure 2). The obtained results showed that 40% and 75% of S. Enteritidis and 33.3% and 100% of S. Typhimurium isolates from milk and hand swabs were positive for spvC gene, respectively (Table 3 and Figure 3).

Discussion

Milk and milk products are implicated in 2-6% of bacterial food-borne outbreaks as reported by surveillance systems from several countries (De Buyser et al., 2001). The level of microbial contamination of raw milk is influenced by several factors such as; the health status of milking cows, hygiene of housing and milking environment and hygiene of milkers (Bramley and Mckinnon, 1990). Milk is considered a good substrate for Staph. aureus growth and enterotoxin production leading to food poisoning which is characterized by nausea, vomiting, abdominal cramps and diarrhea (Bramley and Mckinnon, 1990). Staph. aureus contamination of milk was detected in 17% of bulk tank milk samples (Table 2). Nearly similar results of 18.2% in Turkey (Ekici et al., 2004), 20.8% in Turkey (Aydin et al., 2011) and 21% in Iran (Ahmadi et al., 2009) were previously reported in milk samples collected from dairy farms. Also, Staph. aureus was detected in 10.8% of bulk tank milk samples in Brazil (Fagundes et al., 2010). Higher percentage of 68% in Brazil (De Oliveira et al., 2011) was also documented, this could be due to the examination of milk samples obtained from points of sales not from dairy farms, which in
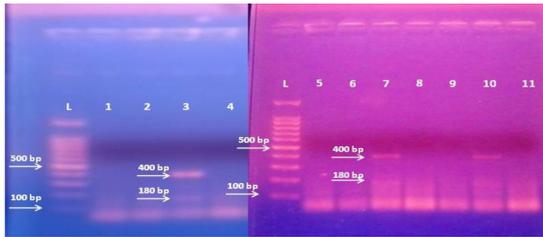
Figure 1. Multiplex PCR results of *Staph. aureus* isolates for the detection of *nuc*, *sea*, *sec* and *sed* genes in 1.2% agarsoe (L: 100 bp ladder, 1: negative control, 3, 7, 10: samples positive for nuc and sea genes, 2, 4-6, 8-9, 11: negative samples)
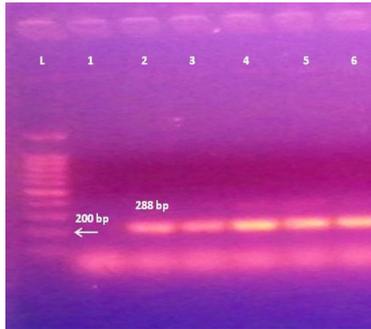

Figure 2. PCR results of *St. agalactiae* isolates for the detection of *cfb* gene in 1.2% agarose (L: 100 bp ladder, 1: negative control, 2-6: samples positive for *cfb* gene)
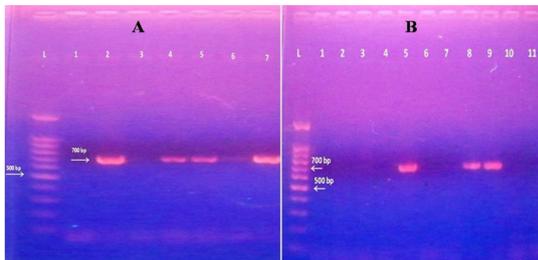

Figure 3. PCR results of *S.* Typhimurium isolates (A) and *S.* Enteritidis isolates (B) for the detection of *spvC* gene in 1.2% agarose (A: [L: 100 bp ladder, 1: negative control, 2, 4, 5, 7: samples positive for *spvC* gene, 3,6: negative samples], B: [L: 100 bp ladder, 1: negative control, 5, 8, 9: samples positive for *spvC* gene, 2-4, 6, 7, 10, 11: negative samples])

turn increase the probability of milk contamination as a result of extra handling and transportation.

In Egypt, Ibrahem (2010) isolated *Staph. aureus* from milk samples at Zagazig, Sharkia province with nearly similar isolation rate (16.5%). However, a higher prevalence of 25% was obtained from milk samples collected from buffalo attending the veterinary hospital in Zagazig, Sharkia (Suelam *et al.*, 2012). Although the study of Suelam *et al.* (2012) was carried out at the same geographical area, the reported prevalence of *Staph. aureus* was higher than that obtained during the current study (17%). This could be attributed to the presence of a source of infection in the examined buffalos which is indicated by high prevalence of *Staph. aureus* in throat swabs

(27.3%) and skin swabs (36.4%) collected from humans dealing with such animals at the veterinary hospital (Suelam *et al.*, 2012). In Aswan, Egypt, a study reported the isolation of the organism from milk samples with the percentage of 34.1% (Abdel-All *et al.*, 2010). Milking equipments are important fomites for transmission of *Staph. aureus* in dairy herds from bovine and human skin to milk (Fox *et al.*, 1991). The spread of *Staph. aureus* throughout the farm especially in milk, farm environment and milking equipments highlights the potential role of such vehicles in transmitting the organism to humans (Jørgensen *et al.*, 2005). Lower *Staph. aureus* isolation rate of 3.6% from milking equipments than the current study (6%) was previously reported (Lee *et al.*, 2012).

About 95% of staphylococcal food poisoning are caused by SE types SEA to SEE (Tamarapu *et al.*, 2001), of which, SEA is considered the most commonly involved enterotoxin (Balaban and Rasooly, 2000). Such toxins are thermoresistant and the ingestion of at least 1 gram of enterotoxin per 100 grams of food is enough to induce food poisoning (Tranter, 1996; Cremonsei *et al.*, 2005). Therefore, the determination of SEs producing strains in food is important with respect to assessing public health risks (Aydin *et al.*, 2011). In the present study, 11.8% of the milk isolates were found encoding *sea* gene (Table 3 and Figure 1). These results are in accordance with 11.7% and 11.6% isolation rates of enterotoxigenic *Staph. aureus* isolates detected in bulk tank milk reported by Neder *et al.* (2011) in Argentina and Morandi *et al.* (2007) in Italy, respectively, while nearly similar to 9.7% reported by Aydin *et al.* (2011) in Turkey. These findings are also in agreement with the frequently reported SEA in dairy products (Normanno *et al.*, 2005; Rall *et al.*, 2008). Higher SEA isolation rate of 21.4% was reported in milk samples collected from Aswan, Egypt (Abdel-All *et al.*, 2010). The detection of the *nuc* gene in the present study in all the positive samples encoding *sea* gene is strongly correlated with enterotoxin production and it is considered as a marker of food contamination with enterotoxigenic *Staph. aureus* (Barski *et al.*, 1996; Tamarapu *et al.*, 2001; Cremonesi *et al.*, 2005).

Milkers' hands are considered as an initial point of contamination with *Staph. aureus* in dairy farms (Olivindo *et al.*, 2009). Approximately, 30-50% of humans carry *Staph. aureus* and one third to one half of the organisms have been shown to be enterotoxigenic (Bergdoll, 1989). *Staph. aureus* was isolated from 10% of milkers' hand swabs and 20% of the isolates were enterotoxigenic encoding for *sea* gene (Table 3 and Figure 1), these results are consistent with those

reported by Ruzickova (1994). The production of SEA is mostly correlated to human strains, thus the contamination of milk during the milking process and handling by milkers could be assumed (Akineden *et al.*, 2008). The isolation of *Staph. aureus* from milkers' hands with the percentages of 3.3% in Brazil (Lee *et al.*, 2012) and 45.9% (Adesiyun *et al.*, 1998) in Trinidad were previously reported. Moreover, an isolation rate of 44.1% was reported in skin swabs of dairy workers in Aswan, Egypt, of which 43.5% were found to encode *sea* gene (Abdel-All *et al.*, 2010). Also, 60% prevalence of *Staph. aureus* was reported from dairy workers in Ismailia, Egypt (Fadel and Ismail, 2009). Several reports of human *Staph. aureus* isolation from milk have been reported suggesting that humans are the source of milk contamination in dairy farms (Roberson *et al.*, 1998; Zadoks *et al.*, 2002; Matyi *et al.*, 2013).

The obtained 4% prevalence rate of *St. agalactiae* from bulk tank milk in the current study (Table 2) was consistent with 3.9% obtained in Portugal (Bexiga *et al.*, 2005) and 3% in USA (Makovec and Ruegg, 2003). Higher prevalence rates of *St. agalactiae* in milk were previously reported in Kenya (Mosabi *et al.*, 1997), Egypt (Amin *et al.*, 2011) and Brazil (Elias *et al.*, 2012). The higher prevalence could be attributed to type of examined milk (mastitic or non-mastitic) and inadequate hygienic and control measures within the examined dairy farms. Although *St. agalactiae* was isolated with low percentage from milkers' hands (Table 2), this result highlights the role of milkers' hands as a source of infection with the bacterium especially when good hygienic measures have not been maintained (Amosun *et al.*, 2010).

The potential for human *St. agalactiae* isolates to be a zoonosis has previously been investigated and results revealed that although human and bovine *St. agalactiae* isolates represent distinct populations, human host associated *St. agalactiae* subtypes may occasionally be transmitted to bovines (Dogan *et al.*, 2005). Moreover, some bovine isolates have been shown to have a genetic relationship with human isolates (Evans *et al.*, 2008). In a previously conducted study, it has been shown that *St. agalactiae* group B serotype III has been incriminated as the major colonizing strain in pregnant women (Ekin and Gurturk, 2006). These findings showed that milk could be a risk factor playing an important role in transmitting some *St. agalactiae* strains with potential zoonoses to humans. Moreover, identical *St. agalactiae* isolates from milk and dairy workers have been reported (Jensen and Aarestrup, 1996).

The pathogenicity of *St. agalactiae* in humans is attributed to several virulence factors; therefore,

in order to investigate the potential of the strains isolated during the current study to pose risk to milk consumers, the presence of CAMP factor specific gene (*cfb*) was investigated. The presence of *cfb* gene in all *St. agalactiae* isolates (Table 3 and Figure 2) indicated the potential of such strains to be virulent causing risk to milk consumers, these results coincide with those reported by Jiusheng *et al.* (2008). It was suggested that the release of CAMP factor during systemic infections could impair the host immune response indicating its rule as a putative virulence factor (Gase *et al.*, 1999).

Salmonella spp. were isolated from 9% of bulk tank milk (Table 2), nearly similar isolation rates of *Salmonella* spp. from milk were previously reported by Van Kessel *et al.* (2003) and Karns *et al.* (2005) in USA. Other studies reported the isolation of *Salmonella* spp with the prevalence of 6.1% in USA (Jayarao and Henning, 2001), 6% in Pennsylvania (Jayarao *et al.*, 2006) and 2.6% in USA (Van Kessel *et al.*, 2004). Higher isolation rates of 28.1% (Van Kessel *et al.*, 2011) and 28.6% (Addis *et al.*, 2011b) were documented in USA and Ethiopia, respectively.

In Egypt, *Salmonella* spp. were isolated from milk samples in Sharkia dairy farms with a higher isolation rate of 28% (Awad, 2002), while another study reported that all of the examined milk samples were free from *Samonella* in Sharkia dairy farms (Ahmed, 2005). The inconsistency between the results of the present study and those aforementioned studies carried out in Sharkia, could be attributed to the difference of the hygienic measures applied in each study area and the use of pooled milk in bulk tanks during the current study while other studies used individual milk samples.

Salmonella species were isolated from 8% of hand swabs of dairy workers (Table 2), this result constitutes public health hazards due to the possibility of transferring the organism to milk and consequently increasing the risk of infection for milk consumers. In Ismailia, Egypt, Fadel and Ismail (2009) isolated *Salmonella* spp. from 3.7% of dairy workers' hands swabs. The probability of fecal contamination of milk, and thus the risk of pathogens transfer appears to be modulated more by farm management and hygiene of dairy workers (Ricci *et al.*, 2013).

Virulence plasmids are one of several *Salmonella* virulence determinants involved in survival and growth in host cells (Finlay and Falkow, 1989). Previous studies have shown that a 7.8-kb region of virulence plasmids consists of five genes described as *spvRABCD* (Gulig *et al.*, 1992). The carriage of *spvC* gene in *Salmonella* isolates seems to increase the growth rate of *Salmonella* spp. in host cells

and affect their interaction with the host immune system (Gulig *et al.*, 1993). *SpvC* gene was found in 75% of *S.* Typhimurium isolates and 40% of *S.* Enteritidis isolates from bulk tank milk (Table 3 and Figure 3). Moreover, the gene was detected in 100% of *S.* Typhimurium isolates and 33.3% of *S.* Enteritidis isolates from hand swabs (Table 3 and Figure 3). *SpvC* gene was previously identified in *S.* Typhimurium isolates of human origin (Swamy *et al.*, 1996; Abouzeed *et al.*, 2000). Moreover, the gene was detected in 100% and 90% of *S.* Enteritidis isolates of bovine and human origin, respectively, in Iran (Amini *et al.*, 2010). The detection of *spvC* in the isolated strains from milk and human sources is of great concern because strains carrying virulence plasmids can cause systemic disease, while those lacking such plasmids can cause local or asymptomatic disease (Heithoff *et al.*, 2008).

Raw milk derived from infected cows with *M. bovis* was regarded as a principle vehicle of infection for humans (Dankner *et al.*, 1993). In the current study, *M. bovis* was isolated from bulk tank milk with the percentage of 1% (Table 2). Similar isolation rate (1%) was reported in bulk tank milk in Brazil (Franco *et al.*, 2013), while nearly similar prevalence rates of 0.7%, 0.8% and 1.6% were also reported in Turkey (Aydin *et al.*, 2012), Brazil (Fujimura Leite *et al.*, 2003) and Tunisia (Ben Kahla *et al.*, 2011), respectively. Although the low isolation rate of *M. bovis* from bulk tank milk samples, the presence of *M. bovis* in milk poses a public health risk. The low prevalence of *M. bovis* could be attributed to the dilution of milk in the bulk tank (Passchyn *et al.*, 2012) and also due to the low number of excreting the organism from infected cows (Gonzalez *et al.*, 1986). Combinations of preventive measures are recommended in dairy farms in order to minimize the risk of milk contamination with potential pathogens. For instance; routine screening of dairy animals and milk, eradication of rats, cleaning and disinfection of farm environment and milking equipments and personal hygienic practices for dairy workers.

Conclusion

In conclusion, the current study reported the detection of potentially virulent bacteria of zoonotic importance in bulk tank milk, milking equipments and dairy workers from some dairy farms in Sharkia Province, Egypt. The presence of such bacteria in raw milk underlines the importance of preventive measures during milking to ensure the quality of milk and to prevent the dissemination of foodborne zoonotic bacteria to consumers. Contaminated milk does not only pose a risk due to its consumption but also it is used in the production of cheese and other dairy products which constitute another potential public health risk. Although the isolation rate of *M. bovis* is low, it is highly recommended to use PCR for confirmation of the bacteria due to high public health risk.

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