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Prevalence and Characterization of *Staphylococcus aureus* Isolated from Bulk Tank Milk Dairy Cow Farms in West Bank-Palestine

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Authors' contributions

This work was carried out in collaboration between both authors. Author GA designed the study, wrote the protocol and the first draft of the manuscript and managed the literature searches. Author HI performed the sample collection and analysis of these samples. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The current study aimed to characterize and document the occurrence of Methicillin-sensitive *S. aureus* (MSSA) and Methicillin-resistant *S. aureus* (MRSA) in bulk tank milk (BTM) samples, farm workers and the environmental surfaces from bovine dairy farms, antibiotic resistance rate, and genetic characterization of clones for both MSSA and MRSA using Enterobacterial Repetitive Intergenic (ERIC-PCR) and Staphylococcal protein A (*spA*) gene typing.
Methodology: A total of 57 bovine BTM samples, 45 samples from farm environmental surfaces and 16 nasal swab samples from farm workers, were collected from 12 bovine dairy farms in the Jenin district in northern West Bank-Palestine, between September and October, 2017.
Results: Results of this research showed that 83.3% of farms had at least one BTM sample contaminated with *Staphylococcus aureus*. Also, 75% and 58.3% of farms had contaminated environmental surfaces and farm worker carriers with *S. aureus*, respectively. Also, it showed that

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58.3% of farms had at least one BTM sample contaminated with MRSA, while 50% of farms had MRSA contaminated environmental surfaces and MRSA farm worker carriers. Results of the current study also showed that 68.4% and 45.6% of bovine BTM samples were contaminated with *S. aureus* and MRSA, respectively. *S. aureus* recovered from different sources showed a high level of resistance to many different antibiotics. Also, results of this study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were multi-drug resistant (MDR). ERIC-PCR profile and *spa* typing showed that some strains of the same clone had been isolated from diverse sources from different farms. This evidence suggested that these strains of the same clone or *spa* type could be circulated between cattle, environment, and humans.

Conclusion: Results of this study showed that Palestine BTM samples are a common source of MRSA. The presence of MRSA isolates in BTM may present a potential public health risk. Therefore, careful monitoring of the resistance status of *S. aureus* in cattle, farm workers, and dairy environmental surfaces is required due to some clones that circulate among them and play a major threat to farm workers who are in close contact with cattle.

Keywords: Bulk tank milk samples; MRSA; S. aureus; bovine dairy farms; Palestine.

1. INTRODUCTION

In humans, *S. aureus* can cause a diverse range of diseases ranging from relatively minor skin infections to serious and life-threatening infections such as endocarditis, pneumonia, and sepsis. In dairy cattle, this pathogen is considered one of the most common causative agents of clinical and sub-clinical mammary infections [1].

Methicillin-resistant *S. aureus* (MRSA) has emerged as a major cause of community-associated (CA-MRSA) and health care-associated (HA-MRSA) infections. In addition, another group of clones called livestock-associated (LA) MRSA has emerged in different countries among different animals [1,2]. The presence of (LA) MRSA strains in bovine milk as well as in bovine dairy farm environments represents a major public health problem for both humans and animals [3,4]. Beta-Lactam agents bind to the penicillin-binding proteins (PBP), which inhibit peptidoglycan crosslink formation, leading to bacterial cell lysis. Resistance against β-lactam antibiotics in MRSA is conferred by the acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene, which encodes PBP2α, which has a low affinity for β-lactam antibiotics. As a result, cell wall synthesis in MRSA strains continues even in the presence of otherwise inhibitory levels of β-lactams [5]. A divergent homolog $mecA_{LGA251}$ has recently been described [6], and now is officially identified as *mecC* [7]. MRSA has increasingly been recognized in farm animal populations in recent years and has been considered as an important pathogen in animals. The prevalence of *S. aureus* as well as MRSA in bovine milk has been reported from several countries such as USA, Denmark [1,8], Turkey [9], Brazil [10], Germany [11], India [12], Iran [13,14], Great Britain [15], Italy [16], and Ethiopia [17]. Antibiotic sensitivity to MRSA and/or MSSA strains isolated from bovine milk has been studied previously [8,9,10,12,15,16,18,19]. In addition, the heterogeneity among MSSA and/or MRSA strains isolated from milk samples has been studied by different methods [1,9,10,11,15,16,18].

Staphylococcal protein A (spA) is considered one of the most important virulence factors encoded by *S. aureus*. This protein plays an essential role by capturing IgG molecules in the inverted orientation and therefore evades phagocytosis. Typing the highly variable Xr region of the *spA* gene is one of the most common methods used to classify *S. aureus* strains and known as *spA* typing. Even if well-established genotyping methods like multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MLEE) are indispensable [20], the *spA*-typing method has advantages due to its high discriminatory power, typing accuracy, speed, reproducibility, and ease of interpretation. It also facilitates communication and data comparison between national and international clinical laboratories [21]. The extracellular domains of spA protein are subject to immune surveillance, which is expected to increase its speed of evolution [22]. Consequently, there are currently 17625 *spA* types in the Ridom SpaServer database (http://spaserver.ridom.de).

The prevalence and characterization of MSSA and MRSA isolated from bovine BTM samples have not been studied previously in Palestine. The current study aimed to characterize and document the occurrence of MSSA and MRSA in

bovine BTM samples, bovine dairy farm environmental surfaces and farm workers, antibiotic resistance rate and genetic characterization of clones for both MSSA and MRSA isolates.

2. MATERIALS AND METHODS

2.1 Sample Collection and *S. aureus* Identification

A total of 57 bovine BTM samples in this study were collected from 12 bovine dairy farms in the Jenin district in northern West Bank-Palestine, between September and October 2017. The total number of cows at these farms was 1343. Machines milked all dairy cattle at these farmmachines. Sixteen nasal swab samples from farm workers and 45 farm environmental samples were collected from farm workers and environmental surfaces, respectively, during the same period of BTM sample collection. During the research period, only one visit was carried out for each farm to collect the samples. Data about the number of cows at each farm and the number of received samples obtained from each farm are presented in Table 1. The swabs used in this study to collect isolates from nasal farm workers and environmental surfaces were from Opti-Swab Collection & Transport System (Amies, Spain). The swabbed ecological surface area was about 50-100 cm^2. Ecological surfaces include machines, cow dung or manure, feeders, ventilation ducts, milking pipelines and farm walls. The samples were immediately refrigerated in a container containing ice cubes and transferred to the Microbiology laboratory at An-Najah National University-Nablus, Palestine, for culturing and identification. Swabbed samples and 150 µl of milk from BTM samples were transferred into tubes containing 7-10 ml Tryptone Soy Broth (TSB) and incubated for 18-24 h at 37ºC. After that, ten µl of the TSB were subcultured by the streaking method on mannitol salt agar. Then, 3-5 separated yellow colonies were subcultured on nutrient agar for further analysis to identify *S. aureus* depending on morphological and biochemical characteristics such as Gram staining, catalase test, and hemolytic reaction and coagulase test.

2.2 Antibiotic Susceptibility Test

Antimicrobial susceptibility for 68 *S. aureus* strains was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method [23]. The antibiotic disks (Oxoid) used in this test were: clindamycin (DA, 2 µg), gentamicin (CN, 10 µg), vancomycin (VA, 30 µg), erythromycin (E, 15 µg), cefoxitin (FOX, 30 µg), sulphamet/trimethop (SXT, 25 µg), ciprofloxacin (CIP, 5 µg), imipenem (IMP, 10 µg), nalidixic acid (NA 30 µg), meropenem (MEM, 10 µg), amikacin (AK, 30 µg), and tetracycline (TE, 30 µg). Mueller Hinton agar (MHA) plates were swabbed with a 4-5-hours-old culture of the bacterial strains, and antibiotic disks were then placed on the inoculated MHA plates. Inhibition zones were measured after incubation of plates at 35ºC for 18-24 h by procedures of the Clinical and Laboratory Standard Institute [23].

The antibiotic cefoxitin was used to detect MRSA. The inhibition zones were determined by procedures of the Clinical and Laboratory Standards Institute [23]. *S. aureus* isolates were considered resistant or sensitive if the inhibition zones were ≤ 21 mm and ≥22 mm, respectively, after incubation on Mueller Hinton agar at 35ºC for 18-24 h. Isolates resistance to three or more drugs were considered multi-drug resistant (MDR). A methicillin resistant control strain (department collection) and a methicillin-susceptible reference strain (*S. aureus* ATCC 25923) were used in this study.

2.3 DNA Extraction and PCR

2.3.1 DNA extraction

The *S. aureus* genome was prepared for PCR according to the method described previously [24]. Briefly, cells were scraped off an overnight cultured nutrient agar plate with a sterile loop, re-suspended with 0.8 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, one mM EDTA [pH 8]), centrifuged for 5 min at 14,500 rpm, and the supernatant was then discarded. Next, the pellet was re-suspended in 0.4 ml of sterile distilled H_2O, boiled for 10 min. After that, the suspension was immediately incubated on ice for 5 min. The debris was pelleted by centrifugation at 14,500 rpm for 5 min. DNA concentration was determined using Nanodrop spectrophotometer (Genova Nano, Jenway) and the DNA samples were stored at -20ºC until use for further DNA analysis.

2.3.2 Amplification of the *mecA* gene

A total of 20 MRSA strains collected from different sample sources (BTM samples, environmental surfaces and nasal swab samples from farm workers) were used to detect the *mecA* gene by PCR. The amplification was carried out by the following specific PCR primers: *mec*A1 (5'-AAA ATC GAT GGT AAA GGT TGG

C-3') and *mec*A2 (5'-AGT TCT GCA GTA CCG GAT TTG C-3'). The amplification product using these primers was 532 bp in length [25]. Each PCR reaction mix (25 µl) was performed using 12.5 µl of PCR premix with $MgCl_2$ (ReadyMix™ 1.5 U Taq PCR Reaction Mix with 0.2 mM dNTP and 1.5 mM $MgCl_2$, Sigma), 0.3 µM of each primer, and 3 µl DNA template. DNA amplification was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation step at 94ºC for 1 min, followed by 40 cycles of initial denaturation 94ºC for 20 s, 52 ºC for 45 s and 72ºC for 30 s ending with a final extension step at 72 ºC for 2 min. After amplification, the PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/ml). A methicillin-resistant control strain (department collection) and a methicillin-susceptible reference strain (*S. aureus* ATCC 25923) were used in this study.

2.3.3 *spA* amplification and phylogenetic tree

The primer sequences used in this study for amplification of the *spA* gene repeated regions were described previously [26]. These primers are spa-1113f (5'-TAA AGA CGA TCC TTC GGT GAG C-3') and spa-1514r (5'-CAG CAG TAG TGC CGT TTG CTT-3'). Each PCR reaction mix (100 µL) was performed using 50 µl of PCR premix with $MgCl_2$ (ReadyMix™ 1.5 U Taq PCR Reaction Mix with 0.2 mM dNTP and 1.5 mM $MgCl_2$, Sigma), 0.3 µM of each primer, and 10 µl of DNA template. DNA amplification was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation (4 min at 94°C), followed by 35 cycles of denaturation 94°C for 40 s, annealing 55°C for 40 s, and extension 72°C for 90 s, with a final extension 72°C for 5 min. A total of 15 µl of amplified PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels and stained with ethidium bromide (0.5 µg/ml). The remaining amount was purified using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the user manual, and sequenced by the dideoxy chain termination method using the ABI PRISM sequencer, model 3130 (Hitachi Ltd, Tokyo, Japan), Bethlehem University, Bethlehem, Palestine. Sequence information was further submitted for accession number in primary bioinformatics web servers. The paper software (http://spatyper.fortinbras.us/) was used for *spA* sequence analysis to determine the clonal types.

The comparison of the continuous sequences was done with previously available sequences of the *spA* gene in the National Center for Biotechnology Information (NCBI) using The Basic Local Alignment Search Tool *(*BLAST*)* system. Multiple sequence alignments were performed using ClustalW of the computer program MEGA software (version 6). The evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analyses were based on alignments obtained from ClustalW of a 223 bp sequence. The phylogenetic tree was constructed using the Neighbor-Joining program using the same software. The robustness of the groupings in the Neighbor-Joining analysis was assessed with 1000 bootstrap re-samplings.

2.3.4 ERIC-PCR typing

The ERIC-PCR was performed using Primer ERIC1: 5`-ATG TAA GCT CCT GGG GAT TCA C-3` and Primer ERIC2: 5-AAG TAA GTG ACT GGG GTG AGC G-3`. Each PCR reaction mix (25 µl) was composed of 10 mM PCR buffer pH 8.3; 3 mM $MgCl_2$; 0.4 mM of each dNTP; 0.8 µM of each primer; 1.5U of Taq DNA polymerase and 3 µl of DNA template. DNA amplification was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 2 min at 94ºC, followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 50ºC for 40 s and extension at 72ºC for 1 min, with a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis on 1.7% agarose gels. The bands in gel images were analyzed using a binary scoring system, which was recorded the absence and presence of bands as 0 and 1, respectively. The binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistical software version 20 (IBM). The clusters of the fingerprints in the constructed dendrogram were described at a 60% similarity level. The number of different bands in each fingerprint was considered for comparison between *S. aureus* strains as previously described [27], based on the following criteria: identical clones (no different bands), "closely related clones" (have 1 different band), "possibility different clones" (have two different bands), "different clones" (have three or more different bands).

3. RESULTS

3.1 Identification of *S. aureus* Isolates

From each sample cultured on mannitol salt agar, 3-5 separated mannitol fermenter colonies (yellow color) were subcultured on nutrient agar and broth for further identification. All isolates showed Gram-positive staining, round shape in clusters, were catalase positive, β-hemolysis on sheep blood agar and were coagulase positive. A total of 251 yellow colonies recovered from all samples were identified as *S. aureus*. These included 136 colonies detected from BTM samples, 35 colonies from nasal swabs from farm workers and 80 provinces from environmental surfaces. Only 68 colonies (isolates), each recovered from one example, were used for further analysis. Results showed that 83.3% (10/12) of farms had at least one BTM sample contaminated with *S. aureus*. Also, 75% and 58.3% of farms had infected environmental surfaces and farm worker carriers with *S. aureus*, respectively. Data about dairy cattle at each farm, number of samples and number of colonies identified as *S. aureus* from these farms are shown in Table 1.

3.2 Antibiotic Resistance

Only 68 of *S. aureus* isolates recovered in this study were selected and tested for antibiotic resistance. Thirty-nine isolates were from BTM samples, 19 were from farm environmental surfaces, and ten were from nasal swabs from farm workers. Isolates recovered from BTM samples showed high resistant to clindamycin, nalidixic acid, tetracycline, meropenem, erythromycin and sulphamet/trimethoprim with a range from 61.5% to 94.8%. These isolates showed more susceptible to ciprofloxacin than other tested antibiotics, where 7.7% of these isolates showed resistant to ciprofloxacin. The results presented that 66.7% of *S. aureus* isolated from BTM samples were identified as MRSA by the cefoxitin disk diffusion method. According to the isolates recovered from farm environmental sources high resistant to nalidixic acid, meropenem, clindamycin, sulphamate/trimethoprim and tetracycline was also demonstrated with a range from 63.2% to 94.7%. These isolates were more sensitive to ciprofloxacin, which showed that approximately 5.3% of *S. aureus* isolates recovered from environmental sources were resistant to ciprofloxacin. Also, these results showed that 57.9% of isolates recovered from farm environmental surfaces were identified as MRSA by the cefoxitin disk diffusion method. Also, *S. aureus* isolated from farm workers had a high rate of resistance to nalidixic acid, meropenem, tetracycline, clindamycin and erythromycin with a range between 50% and 80%. These isolates were more sensitive to amikacin, imipenem, ciprofloxacin, which showed that 10% of them were resistant to these antibiotics. The current results also showed that MRSA was detected in 30% of *S. aureus* isolated from nasal swabs from farm workers by the cefoxitin disk diffusion method. The antibiotic resistance profile of 68

Table 1. The number of dairy cattle at each farm, the number of samples obtained from each farm and the number of *S. aureus* colonies recovered from these samples at each farm

Farm	No. of cows	No. of samples			No. of *S. aureus* colonies			Total N
		BTM*	En	H	BTM	En	H	
A	60	8	3	2	26	4	0	30
B	17	8	3	2	7	8	8	23
C	5	2	2	1	4	0	0	4
D	60	3	3	1	3	0	4	7
E	18	2	3	1	8	7	4	19
F	85	4	3	1	16	8	4	28
G	60	4	3	1	0	4	0	4
H	300	2	3	1	4	0	0	4
I	200	10	6	2	32	21	3	56
J	8	3	4	1	0	6	0	6
K	80	5	6	1	20	16	4	40
L	450	6	6	2	16	6	8	30
Total	1343	57	45	16	136	80	35	251

** BTM: Bulk tank milk sample; En: Environmental surface sample; H: nasal swab sample from farm worker; N: Number of colonies*

S. aureus isolates recovered from different sample sources is presented in Table 2. It was found that 20 MRSA isolates detected by the cefoxitin disk diffusion method carried the *mecA* gene. Also, results in the current study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were MDR.

Results of this study showed that 58.3% (7/12) of farms had MRSA contaminated BTM samples, while 50% (6/12) of farms had MRSA contaminated environmental surfaces and MRSA farm worker carriers. Results also showed that BTM samples, environmental surfaces and nasal swabs from farm workers from 4 farms (C, D, G, and H) were MRSA negative. Distribution of MRSA and MSSA isolates according to the farms and source of samples is presented in Table 3. Results of this study also showed that 68.4% and 45.6% of bovine BTM samples were contaminated with *S. aureus* and MRSA, respectively.

3.3 ERIC-PCR Analysis

ERIC-PCR typing of 54 *S. aureus* isolates recovered from BTM samples, environmental surfaces of cow dairy farms and nasal swab samples from farm workers were clustered into six groups (clusters) at a 60% similarity level. The profile of ERIC-PCR typing showed that there are five identical clones (IC), and 11 closely related clones (CRC) circulating among these farms. The ICs recognized in this study were: IC1 with strains 11, 21, 45, 47 and 48; IC2 with strains 25 and 26; IC3 with 1 and 34; IC4 with strains 35 and 36 and IC5 with strains 13, 14, 42 and 49. For example, in case of IC1, the strains of this clone were identified from BTM samples, and environmental sample from farms D, G and I. Another example, the pressures of IC5 were identified from BTM samples, environmental samples and a nasal swab from a farm worker from farm E and I. According to CRCs, it included CRC1 to CRC11. For example, in the case of CRC1, the ERIC-PCR profile of strain 44 had one band difference in comparison with the ERIC-PCR profile of strains 11, 21, 45, 47 and 48. Here in Fig. 1, we mentioned the source of samples and the name of farms for ICs only. The clusters (Cs), ICs and CRCs of 54 *S. aureus* isolates generated by ERIC-PCR are presented in Fig. 1.

3.4 Detection *spA* Type and Phylogenetic Tree

Eighteen of *spA* PCR products were sequenced to determine the types of *spA* circulated in bovine dairy farms in Palestine. The sequences of *spA* genes in this study were examined to detect repeat regions and to identify the particular *spA* type using paper software (http://spatyper.fortinbras.us/). The sequences of *spA* genes (n=18) were further registered at the GenBank database under the accession numbers (MG759487-MG759504). Four out of 18 *spA* sequences, which have accession numbers (MG759487), (MG759491), (MG759494) and (MG759504) belong to *spA* type t164, and two other *spA* sequences which have accession numbers (MG759500) and (MG759503) belong to t9129 and t2518, respectively. The type of twelve other *spA* sequences could not be determined. Three *spA* sequences, which have the *spA* type t164 were recovered from BTM samples from different farms (I, K and F), while the fourth was recovered from a nasal swab sample from a farm worker who was working at farm I. The *spA* sequence, which has accession number (MG759488) is closely related to *spA* sequences belonging to type t164. This *spA* sequence shows a difference in one repeated sequence. Also, the t164 *spA* sequence was recovered from a BTM sample from farm I. All *spA* gene sequences obtained in this study and other similar *spA* sequences retrieved from GenBank were used to construct the phylogenetic tree (Fig. 2). Depending on the phylogenetic tree, the *spA* sequences, which have accession numbers (MG759497) and (MG759489) could be identical or very closely related clones. These *spA* sequences were obtained from *S. aureus* recovered from BTM samples from farm L and farm I, respectively. According to *spA* sequences, which have accession numbers (MG759493) and (MG759487) were clustered together in the phylogenetic tree. Even the *spA* sequence, which has the accession number (MG759487) belongs to the type t164, while the type of the *spA* sequence, which has the accession number (MG759493) could not be determined. These *spA* sequences are very closely related, although they show differences in one repeated sequence. S*pA* sequences with accession numbers (MG759493) and (MG759487) were recovered from BTM sample and nasal swab sample from farm worker from farm K and farm I, respectively.

Table 2. Antibiotic resistance profiles of 68 *S. aureus* isolates recovered from different sample sources obtained from bovine dairy farms

Antibiotic	Antibiotic resistance** No. (%)								
	BTM* (no. of isolates=39)			H (no. of isolates=10)			En (no. of isolates=19)		
	S	I	R	S	I	R	S	I	R
DA	8 (20.5)	3 (7.7)	28 (71.8)	2 (20)	3 (30)	5 (50)	3 (15.8)	2 (10.5)	14 (73.7)
CN	17 (43.6)	1 (2.6)	21 (53.8)	7 (70)	1 (10)	2 (20)	10 (52.6)	4 (21.1)	5 (26.3)
VA	19 (48.7)	0 (0.0)	20 (51.3)	8 (80)	0 (0)	2 (20)	13 (68.4)	0 (0.0)	6 (31.6)
E	11 (28.2)	4 (10.3)	24 (61.5)	3 (30)	2 (20)	5 (50)	7 (36.8)	3 (15.8)	9 (47.4)
FOX	13 (33.3)	-	26 (66.7)	7 (70)	-	3 (30)	8 (42.1)	-	11 (57.9)
SXT	11 (28.2)	1 (2.6)	27 (69.2)	6 (60)	0 (0)	4 (40)	4 (21.1)	3 (15.8)	12 (63.1)
CIP	22 (56.4)	14 (35.9)	3 (7.7)	8 (80)	1 (10)	1 (10)	13 (68.4)	5 (26.3)	1 (5.3)
IMP	18 (46.2)	3 (7.7)	18 (46.2)	9 (90)	0 (0)	1 (10)	8 (42.1)	1 (5.3)	10 (52.6)
NA	1 (2.6)	1 (2.6)	37 (94.8)	1 (10)	1 (10)	8 (80)	1 (5.3)	0 (0.0)	18 (94.7)
MEM	6 (15.4)	3 (7.7)	30 (76.9)	3 (30)	0 (0)	7 (70)	3 (15.8)	0 (0.0)	16 (84.2)
AK	21 (53.8)	1 (2.6)	17 (43.6)	9 (90)	0 (0)	1 (10)	10 (52.6)	1 (5.3)	8 (42.1)
TE	8 (20.5)	1 (2.6)	30 (76.9)	4 (40)	0 (0)	6 (60)	4 (21.1)	2 (10.5)	13 (68.4)

***No.: number of isolates; S: Susceptible; I: Intermediate; R: Resistant*

DA: Clindamycin; CN: Gentamycin; VA: Vancomycin; E: Erythromycin; FOX: Cefoxitin; SXT: sulphamet/trimethop; CIP: Ciprofloxacin; IMP: Imipenem; NA: Nalidixic acid; MEM: Meropenem; AK: Amikacin; TE: Tetracycline

**BTM: S. aureus isolated from Bulk tank milk sample; En: S. aureus isolated from Environmental surface sample; H: S. aureus isolated from nasal swab sample from farm worker*

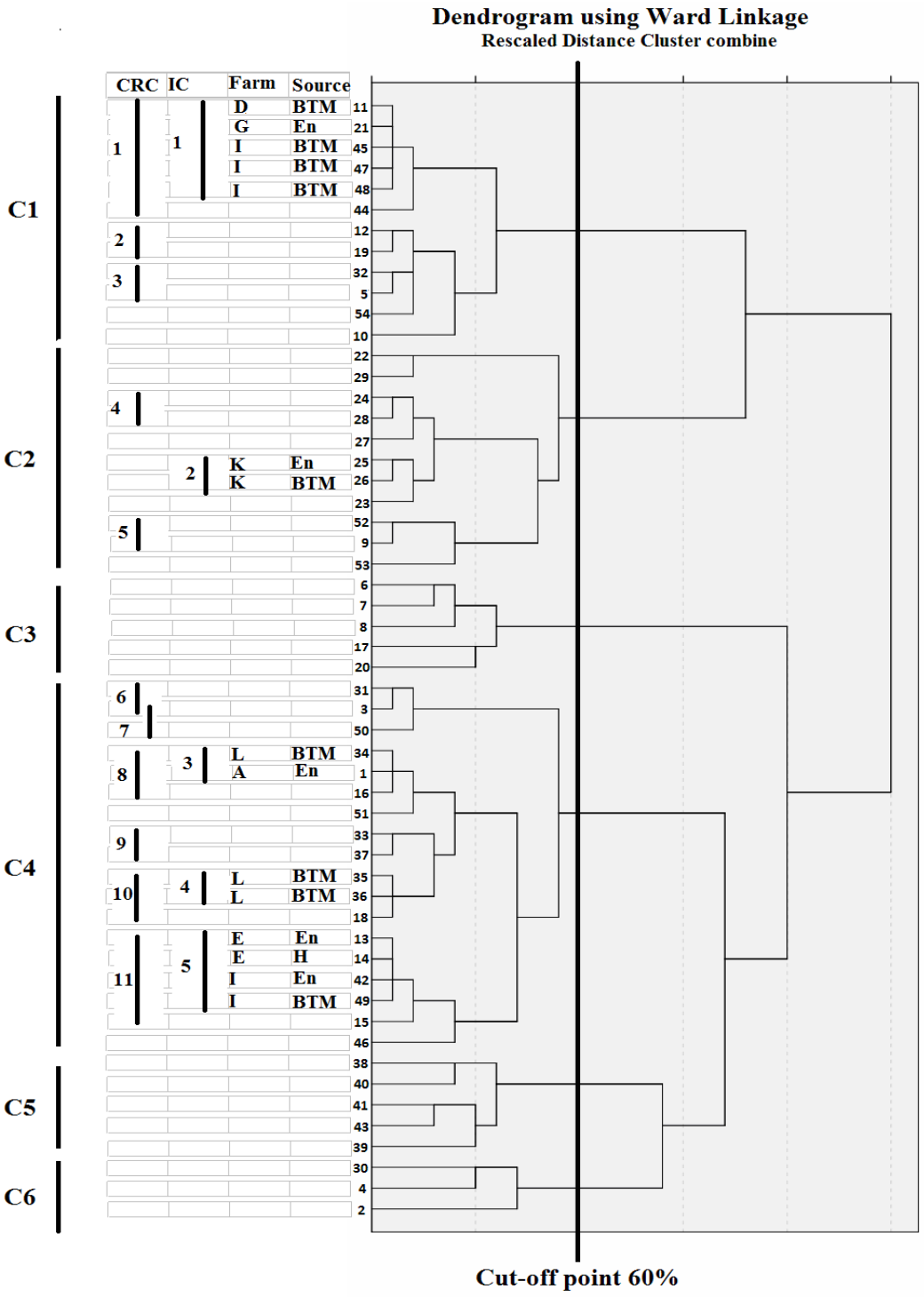

Fig. 1. Dendrogram of 54 *S. aureus* isolates recovered from different sample sources based on the UPGMA method derived from analysis of the ERIC-PCR profiles at a 60% similarity level

C: Cluster; IC: Identical clone; CRC: Closely related clone; BTM: Bulk tank milk sample; H: Nasal swab sample from farm workers; En: Environmental surface sample

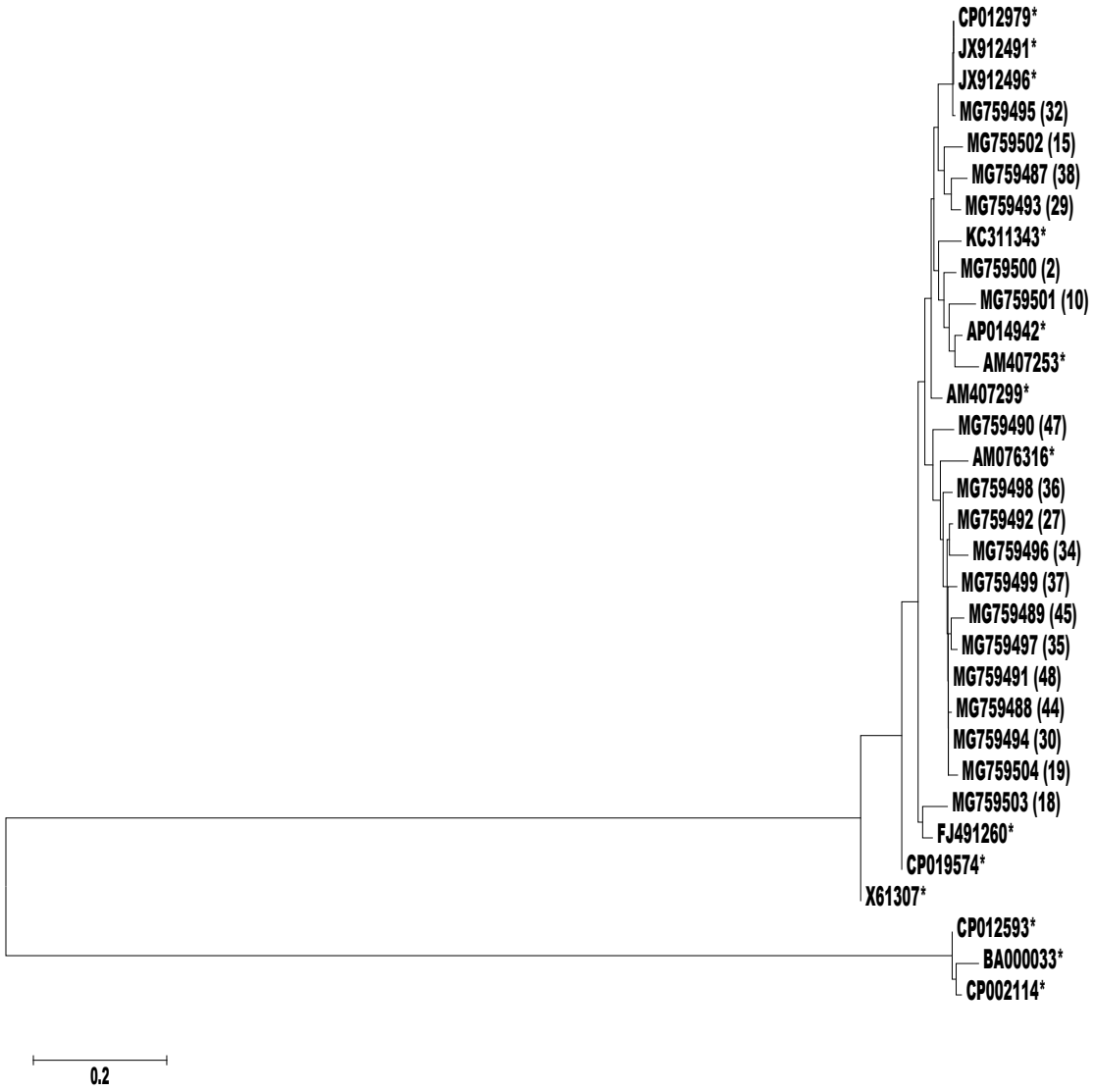

Fig. 2. Phylogenetic tree constructed using the Neighbor-Joining (N-J) method based on partial *spA* gene sequences. Reference *spA* sequences were retrieved from GenBank and denoted by asterisks. The tree was bootstrapped with 1000 resamplings, and the corresponding genetic distance is indicated by a bar. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA6. Numbers in brackets near the GenBank accession numbers denoted that these spA sequences on the phylogenetic tree have the same strain numbers on the dendrogram

4. DISCUSSION

The emergence of MRSA in cattle is considered one of the major public health problems. This type of pathogen can contaminate products of animal origin and can cause infection to humans as well as animals. It has been reported that this pathogen can transmit from farm animals such as cattle to farm workers [28].

The result of the current study showed a high occurrence of MRSA in bovine dairy farms in Northern West Bank-Palestine, 58.3% (7/12) of farms had at least one BTM sample contaminated with MRSA. This percentage is higher than in other studies carried out previously in Germany and USA, which showed that the herd prevalence of MRSA had a range from 4% to 16.7% [1,11]. Also, *S. aureus* was detected in

Table 3. Distribution of MRSA and MSSA isolates according to the farms and sample sources

Farm	Number of *S. aureus* isolates	*S. aureus* isolates			
		MRSA isolates		MSSA isolates	
		BTM* n (%)	H and En n (%)	BTM n (%)	H and En n (%)
A	9	7 (77.8)	0 (0.0)	1 (11.1)	1 (11.1)
B	7	2 (28.6)	1 (14.3)	1 (14.3)	3 (42.8)
C	1	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
D	2	0 (0.0)	0 (0.0)	1 (50)	1 (50)
E	5	1 (20)	0 (0.0)	1 (20)	3 (60)
F	7	4 (57.1)	2 (28.6)	0 (0.0)	1(14.3)
G	1	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)
H	1	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
I	14	5 (35.7)	3 (21.4)	3 (21.4)	3 (21.4)
J	2	0 (0.0)	2 (100)	0 (0.0)	0 (0.0)
K	10	3 (30)	3 (30)	3 (30)	1 (10)
L	9	2 (22.2)	1 (11.2)	3 (33.3)	3 (33.3)

**BTM: Bulk tank milk sample; H: Nasal swab sample from farm worker; En: Environmental surface sample*

68.4% of bovine BTM samples in this study. The results in this study were consistent with previous reports from USA, Denmark, and Norway, where the occurrence of *S. aureus* in bovine BTM samples was 60%, 55% and 75%, respectively [8,29]. However, the results were in contrast to other studies from Iran, Brazil, and Ethiopia, where the occurrence of *S. aureus* in bovine BTM samples was 16%, 10.8% and 38%, respectively [10,13,17]. In this study, results showed that MRSA was detected in 45.6% of bovine BTM samples. The percentage was higher than in previous studies from USA, Italy, and Ethiopia, where the occurrence of MRSA in bovine BTM was 1.3%, 3.8%, 26%, respectively [1,16,17]. The possible explanation for the high MRSA occurrence in bovine BTM samples and herds may be due to several reasons. These reasons include the use of antimicrobial agents frequently, higher levels of contact between animals during shipment, differences in farming practices, Inadequate or improper disinfecting milking machines, high cattle population density in these farms, and lack of special places for isolation of sick or infected animals at most of these farms. Also, variations in methodological approaches used in these studies have variable sensitivity to detect and isolate these organisms.

The results of this study demonstrate that *S. aureus* recovered from BTM samples had a high level of resistance to many different antibiotics. It was in agreement with other previous studies reported from several countries such as Turkey, India, and Italy [9,12,16]. The results from the current study showed that 100% and 69.2% of MRSA and MSSA strains, respectively, isolated from BTM samples were MDR. These results were consistent with studies previously published from Italy and Turkey, which showed a prevalence of MDR 90.6% and 100%, respectively [9,16]. On the other hand, these results were in contrast to a report published previously from the USA, which showed an MDR of prevalence 5.4% [1]. The high level of resistance against many different antibiotics as well as the high level of MDR may be due to the use of antimicrobial agents on dairy farms as preventive measures during dry cow therapy as well as used in animal food production systems as additives [30]. Moreover, antimicrobial susceptibility testing is not performed before treatment of infected animals.

In this study, ERIC-PCR fingerprinting profiling and *spA* typing showed that some ERIC-PCR fingerprinting profiles or *spA* sequences for certain strains belonged to the same clones. The strains of the same clone had been isolated from different sample sources and different farms. For example, three *spA* sequences which have the *spA* type t164 were recovered from BTM samples from different farms (I, K and F), while the fourth *spA* sequence was recovered from a nasal swab from a farm worker who was working at the farm I. The *spA* sequence which has the accession number (MG759488) is closely related to the *spA* sequences, which belonged to type t164. This *spA* sequence had a difference in one repeated sequence, which may be due to a mutation in that repeated sequence. According to the phylogenetic tree, the strains which have accession numbers (MG759493) and (MG759487) are clustered together, even the strain which has accession number (MG759487) belongs to the type t164. These strains are very

closely related and differed in one repeated sequence, which may be due to a mutation in that repeated sequence. The strains which have the accession numbers (MG759493) and (MG759487) were recovered from a BTM sample and nasal swabs from farm workers from farm K and farm I, respectively. According to the ERIC-PCR profiles, there were 5 ICs isolated from BTM samples, environmental sources and nasal swabs from farm workers. Also, the strains of the same clone, which had indistinguishable ERIC-PCR profiles, were circulated at more than one bovine dairy farm. Eleven CRCs isolated from different sample sources were also detected. The strains of these CRCs, which have closely-related fingerprint patterns circulated at different farms. The strains which had closely-related ERIC-PCR fingerprint patterns were probably derived from a single parental organism but possibly have changed over a long period. This change may be due to some factors such as mutations due to deletion or replacement of nucleotides, activation of transposons, developed mutations caused by defect in DNA repairing system and genomic recombinants caused by genomic alteration [31].

Pulsed-field gel electrophoresis (PFGE) is accepted as a “gold standard” for typing several Gram-positive and Gram-negative bacteria. It is considered a highly discriminatory and sensitive technique for distinguishing strains of *S. aureus*. However, this technique requires expertise, a special instrument, and takes time and effort to conduct [32]. PCR-based typing methods provide a feasible alternative tool, which is more rapid and cost-effective than other molecular typing systems [33]. The repetitive sequence-based PCR method is considered one of the molecular epidemiologic typing techniques, which enables the generation of DNA fingerprinting that can discriminate bacterial strains. This method is simple and rapid for distinguishing closely related strains to explain phylogenetic relationships between strains. Three types of repetitive sequences can be studied using molecular epidemiologic techniques, including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and box element sequences [31]. Sequenced-based typing methods have been developed and are now widely used, such as multilocus sequence typing (MLST) and *spA* typing, which are most frequently used for *S. aureus*. DNA sequence-based typing methods, especially the *spA*-typing method, have advantages due to the high discriminatory power, typing accuracy, speed, reproducibility, ease of interpretation, generation of unambiguous and portable data, amenability to create central databases, which facilitates communication and enables the data comparison between national and international clinical laboratories [21,34].

These results support that the presence of MRSA strains in both bovine milk and dairy farm environments present a major threat to farm workers who are in close contact with cattle, veterinarians, and animals that are exposed to infected cattle [3,4]. This evidence suggested that these strains of the same clone or *spA* type could be circulated between cattle and humans. The transmission of this *spA* type between two hosts (humans and cattle) may be due to adaptation. This result was in agreement with other previous studies which showed that some strains were circulated between humans and cattle [9,19,35].

5. CONCLUSION

This study characterized *S. aureus* isolated from BTM samples, environmental sources and humanitarian workers in close contact with these bovine dairy farms. Results of this study showed that Palestine BTM samples are a familiar source of MRSA. The presence of MRSA isolates in BTM may present a potential public health risk. Therefore, careful monitoring of the resistance status of *S. aureus* in cattle, farm workers, and dairy environmental surfaces is required, due to some clones that circulate among them and play a significant threat to farm workers who are in close contact with cattle.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Haran KP, Godden SM, Boxrud D, Jawahir S, Bender JB, Sreevatsan S. Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. J Clin Microbiol. 2012;50(3):688-695.
2. Köck R, Schaumburg F, Mellmann A, Köksal M, Jurke A, Becker K, Friedrich AW. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) as causes of human infection and

colonization in Germany. PLoS One. 2013;8(2):e55040.
DOI: 10.1371/journal.pone.0055040

3. Lee JH. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol. 2003;69:6489-6494.
4. Juhász-Kaszanyitzky E, Jánosi S, Somogyi P, Dán A, van der Graaf-van Bloois L, van Duijkeren E, et al. MRSA transmission between cows and humans. Emerg Infect Dis. 2007;13:630-632.
5. Gopal S, Divya KC. Can methicillin-resistant *Staphylococcus aureus* prevalence from dairy cows in India act as potential risk for community-associated infections?: A review. Vet World. 2017; 10(3):311.
6. García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD, et al. Methicillin resistant *Staphylococcus aureus* with a novel *mecA* homolog in human and bovine populations in the UK and Denmark: A descriptive study. Lancet Infect Dis. 2011;11:595–603.
7. Ito T, Hiramatsu K, Tomasz A, de Lencastre H, Perreten V, Holden MT, et al. Guidelines for reporting novel mecA gene homologues. Antimicrob Agents Chemother. 2012; 56(10):4997-4999.
8. Sato K, Bennedsgaard TW, Bartlett PC, Erskine RJ, Kaneene JB. Comparison of antimicrobial susceptibility of *Staphylococcus aureus* isolated from bulk tank milk in organic and conventional dairy herds in the midwestern United States and Denmark. J Food Prot. 2004;67(6):1104-1110.
9. Turkyilmaz S, Tekbiyik S, Oryasin E, Bozdogan B. Molecular epidemiology and antimicrobial resistance mechanisms of methicillin-resistant *Staphylococcus aureus* isolated from bovine milk. Zoonoses Public Health. 2010;57:197-203.
10. Fagundes H, Barchesi L, Filho AN, Ferreira LM, Oliveira CA. Occurrence of *Staphylococcus aureus* in raw milk produced in dairy farms in São Paulo state, Brazil. Braz J Microbiol. 2010;41(2):376-380.
11. Spohr M, Rau J, Friedrich A, Klittich G, Fetsch A, Guerra B, Hammerl JA, Tenhagen BA. Methicillin-resistant *Staphylococcus aureus* (MRSA) in three dairy herds in southwest Germany. Zoonoses Public Health. 2011;58:252-261.
12. Kumar R, Yadav BR, Singh RS. Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitic Sahiwal cattle. J Biosci. 2011;36: 175-188.
13. Rahimi E, Alian F. Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk. Vet Arhiv. 2013;83(1):23-30.
14. Khaji L, Shahreza MHS. *SCCmec* types in Methicillin-Resistant *Staphylococcus aureus* Strains of Various Types of Milk. Electron J of Biol. 2017;13(1):14-17.
15. Paterson GK, Morgan FJ, Harrison EM, Peacock SJ, Parkhill J, Zadoks RN, et al. Prevalence and properties of *mecC* methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine bulk tank milk in Great Britain. J Antimicrob Chemother. 2014; 69(3):598-602.
16. Cortimiglia C, Luini M, Bianchini V, Marzagalli L, Vezzoli F, Avisani D, et al. Prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. aureus* clonal complexes in bulk tank milk from dairy cattle herds in Lombardy region (Northern Italy). Epidemiol Infect. 2016;144(14): 3046-3051.
17. Tolosa T, Verbeke J, Piepers S, Tefera M, Getachew Y, Supré K, et al. Milk production, quality, and consumption in Jimma (Ethiopia): Facts and producers', retailers', and consumers' perspectives. Prev Vet Med. 2016;124:9-14.
18. Huber H, Koller S, Giezendanner N, Stephan R, Zweifel C. Prevalence and characteristics of meticillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland, 2009. Euro Surveill. 2010;15(16):Pii: 19542.
19. Hata E, Katsuda K, Kobayashi H, Uchida I, Tanaka K, Eguchi M. Genetic variation among *Staphylococcus aureus* strains from bovine milk and their relevance to methicillin-resistant isolates from humans. J Clin Microbiol. 2010;48:2130-2139.
20. Koreen L, Ramaswamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN. *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J Clin Microbiol. 2004;42(2):792-799.
21. Votintseva AA, Fung R, Miller RR, Knox K, Godwin H, Wyllie DH, et al. Prevalence of *Staphylococcus aureus* protein A (spa)

mutants in the community and hospitals in Oxfordshire. BMC Microbiol. 2014;14:63. DOI: 10.1186/1471-2180-14-63

22. Stephens AJ, Inman-Bamber J, Giffard PM, Huygens F. High-resolution melting analysis of the spa repeat region of *Staphylococcus aureus*. Clin Chem. 2008;54(2):432-436.
DOI: 10.1373/clinchem.2007.093658
23. Clinical and Laboratory Standards Institute (CLSI), Performance standards for antimicrobial susceptibility testing. 26th ed. CLSI supplement. M100S. Wayne, PA, USA; 2016.
24. Adwan G, Adwan K, Jarrar N, Salama Y, Barakat A. Prevalence of *seg, seh* and *sei* genes among clinical and nasal *Staphylococcus aureus* isolates. Br Microbiol Res J. 2013;3(2):139-149.
25. Adwan G, Adwan K, Jarrar N, Amleh A. Molecular detection of nine antibiotic resistance genes in methicillin resistant *Staphylococcus aureus* isolates. Roum Arch Microbiol Immunol. 2014;73(1-2):9-17.
26. Prosperi M, Veras N, Azarian T, Rathore M, Nolan D, Rand K, et al. Molecular epidemiology of community-associated methicillin-resistant *Staphylococcus aureus* in the genomic era: A cross-sectional study. Sci Rep. 2013;3:1902.
27. Adwan G, Shtayah A, Adwan K, Al-Sheboul S, Othman S. Prevalence and molecular characterization of *P. aeruginosa* isolates in the West Bank-Palestine for ESBLs, MBLs and integrons. J Appl Life Sci Int. 2016;8(2):1-11, Article no. JALSI.29259.
DOI: 10.9734/JALSI/2016/29259
28. Caruso M, Latorre L, Santagada G, Fraccalvieri R, Miccolupo A, Sottili R, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) in sheep and goat bulk tank milk from Southern Italy. Small Rumin Res. 2016;135:26-31.
29. Jørgensen HJ, Mørk T, Høgåsen HR, Rørvik LM. Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J Appl Microbiol. 2005;99(1):158-166.
30. Piddock LJV. Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic resistant bacteria that infect man and compromise antimicrobial chemotherapy? J Antimicrob Chemother. 1996;38:1-3.
31. Moosavian M, Darban D. Genomic fingerprints analysis of coagulase-positive and negative Staphylococci isolated from patients with bacteremia by repetitive sequence based PCR method. Afr J Microbiol Res. 2010;4(5):354-359.
32. Çiftci A, Onuk E, Findik A, Yildirim T, Sogut MU. Molecular typing of *Staphylococcus aureus* strains from bovine mastitis by pulsed-field gel electrophoresis and polymerase chain reaction based on coagulase and Protein A gene polymorphisms. J Vet Diagn Investigation, 2009;21(6):849-853.
33. Wilailuckana C, Tribuddharat C, Tiensasitorn C, Pongpech P, Naenna P, Rugdeekha S, et al. Discriminatory powers of molecular typing Techniques for methicillin-resistant *Staphylococcus aureus* in a university hospital, Thailand. Southeast Asian J Trop Med Public Health. 2006;37(2):327-334.
34. Faria NA, Carrico JA, Oliveira DC, Ramirez M. De Lencastre H. Analysis of typing methods for epidemiological surveillance of both methicillin resistant and methicillin-susceptible *Staphylococcus aureus* strains. J Clin Microbiol. 2008;46:136-144.
35. Basanisi MG, La Bella G, Nobili G, Franconieri I, La Salandra G. Genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk and dairy products in South Italy. Food Microbiol. 2017;62:141-146.
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