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Genetic Sequence and phylogenetic analysis of some virulence genes of *Staphylococcus aureus* isolated from dairy farms and human in Qalyobia Governorate, Egypt

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ABSTRACT

Staphylococcus aureus is a commensal organism of skin and nose in nearly 30% of the human population. It produces several virulence factors like biofilm formation regulated by bap gene encodes for biofilm-associated protein. In addition to *Staphylococcus aureus* efflux-mediated multidrug resistance gene smr. Three virulence genes were identified using conventional PCR on 12 *Staphylococcus aureus* strains. The most commonly found virulence genes were nuc12/12 (100%), smr12/12 (100%), and bap10/12 (83.33%). Three favorable PCR products from each *S. aureus* bap and smr gene were used for sequencing and phylogenetic analysis using the Bio Edit 7.0.4.1 and MUSCLE programs. Then Six nucleotide sequences for *S. aureus* isolates were produced and entered into GenBank using accession numbers OR344353 toOR344355 for bap gene, OR344356to OR344358 for smr gene, and showed almost typical amino acid sequences of *S. aureus* isolates from bovine milk and dairy utensils. In contrast, human isolates showed major mutations through change and addition. The phylogenetic tree targeting the bap gene formed four clades where all *S. aureus* isolates recovered from bovine milk and dairy utensils revealed a high degree of similarity to Staphylococcus epidermidis isolated from nosocomial infections in humans in Brazil and low homology with *S. aureus* isolate from bovine milk in India. Also, *S. aureus* isolated from human nasal swabs in Egypt showed a high homology with *S. aureus* isolates isolated from bovine milk in India. Concerning the smr gene, all *S. aureus* isolates from this study showed a high homology with S. epidermidis isolated from ovine milk in Italy.

1. INTRODUCTION

Staphylococcus aureus is a well-known nosocomial, community, and livestock-associated bacterial pathogen in humans and animals (Rao *et al*., 2022). In both humans and animals, it causes diseases. Due to *S. aureus* ability to form biofilms and the rise in drug-resistant strains, these illnesses are more common and challenging to treat (Oliveira *et al*., 2018). *Staphylococcus aureus (S. aureus*) contamination of milk resulted from an infection of the udder or from unsanitary conditions during or after milking, and these events were caused by human action (Rehman *et al*., 2014). A biofilm is an extracellular matrix (ECM) that resembles a membrane and is made up of extracellular polymeric substances (EPS), including nucleic acid and polysaccharides, and bacteria release proteins as they grow. Biofilms are organized colonies of bacteria (Karygianni *et al*., 2020). The interaction between EPS and bacterial aggregation provides the adhesion and viscosity of biofilm. Bacteria can thus adhere to biotic and abiotic surfaces (Di Martino, 2018). Polysaccharide intercellular adhesion (PIA) is an essential element in *S. aureus* biofilm development among the polymeric molecules (EPS) implicated in ECM, PIA plays a critical role in staphylococcal biofilm production and immune evasion via proteins expressed by the intercellular adhesion (*ica*ADBC) operon in the *ica* locus (Nguyen *et al*., 2020). Other processes are not dependent on the *ica* operon. *The S. aureus bap* gene encodes a surface protein called bap (biofilm-associated protein). During biofilm formation, bap was discovered as the primary determinant of successful surface adherence and intercellular adhesion (Cucarella *et al*., 2004). All *S. aureus* strains containing the *bap* gene exhibit high adhesion and significant biofilm-forming capacity, Bap's N-terminal region is released into (ECM) and organized into amyloid fibers to aid in the formation of the *S.*

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aureus biofilm, *bap* increases epithelial cell adhesion during infection to promote persistence in the mammary gland and binds to host receptors (Taglialegna *et al*., 2016). It causes intra-mammary infection because biofilms protect against the host immune system and antibiotics, which are essential in eradicating infections (Gomes *et al*., 2016). By enclosing themselves in or on the surface of a substrate using self-produced exopolysaccharides (biofilm matrix) (Römling *et al.,* 2014). And results in chronic persistent infection (Guilhen *et al*., 2017). A significant contributor to multiple drug resistance is multidrug efflux pumps. These actively expel antibiotic substances from bacterial cells and are found in the biological membrane of the bacteria (Andersen *et al.,* 2015). Multiple groups discovered the efflux pump gene smr in multiple plasmids that conferred ethidium bromide and antiseptic resistance in the late 1980s. The gene's designation was ebr (Ethidium bromide resistance gene) (Sasatsu *et al*., 1989), *qacC/D* (gene encodes for quaternary ammonium compounds (Littlejohn *et al*., 1991) or *smr* (Staphylococcal multidrug resistance gene) (Grinius *et al*., 1992), However, sequencing analysis showed that all of the determinants mentioned by these authors were the same. This gene encodes the efflux pump and is present in both *S. aureus* and coagulase-negative staphylococci (CoNS) on small and large plasmids, encoding the *same* efflux pump (Littlejohn *et al*., 1991). Smr forms a pore-like structure through which the substrate can flow and conveys poor resistance against chemicals, including quaternary ammonium compounds like benzalkonium chloride and Ethidium bromide (Yamada *et al*., 2006). The nuc gene encodes a heat-stable thermonuclear that is only found in *S. aureus* and not in (CoNS) (Canning *et al*., 2020).

The study's goal is to confirm *S. aureus* isolates that were isolated from dairy animal farms and humans by molecular amplification of the *nuc* gene, followed by the measurement of biofilm formation and antiseptic resistance (*bap* and *smr*) genes using multilocus sequence typing (MLST) and estimates their evolutionary relationships.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The current study was conducted on 12 *S. aureus* isolates from 304 samples. The whole twelve *S. aureus* isolates in our recent study include the following: Eight isolates isolated from dairy animals and utensils originate from four different dairy farms in Qalyobia Governorate (2 isolates from raw cow milk, two nasal swabs, one from cow and other from buffalo, two teat swabs one from cow and other from buffalo and two from dairy utensils). In addition, four isolates from dairy workers (2 isolates from hand swabs and two isolates from nasal swabs), according to Singh *et al*. (2008).

2.2 Molecular identification of S. aureus

2.2.1DNA extraction

Using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) and the manufacturer's instructions, DNA was extracted from bacterial cultures obtained according to Singh *et al*. (2008) and DNA extraction according to Sambrook *et al*. (1989).

2.2.2 Molecular identification using conventional Polymerase Chain Reaction (PCR)

For the identification of *S. aureus* targeting *the nuc* gene, The GS-96 gradient thermal cycler (hercuvan, Malaysia) was used to perform the PCR reaction. The final volume of the response was 25 µl, and it contained 12.5 µl of the 2x MyTaq Red Mix Master Mix (Cat. BIO-25043, Meridian Bioscience, UK), 0.5 µl (10 µM) of each primer, one µl of the target DNA, and 10.5 µl DNA grade water. The PCR products were separated by electrophoresis on 1.5% agarose gel and then photographed and analyzed by the InGenius3 gel documentation system (Syngene, UK). The used primers are listed in Table (1). The antiseptics resistance and biofilm formation Genes were detected in all *S. aureus* isolates: smr (qac antiseptics resistance gene) and bap (biofilm formation gene). Table (2) shows the primers applied and the cycle conditions

Table (1): PCR primers and probes

Gene.	Sequence(5´-3´)	Amplicon size(bp)	Reference
nuc	F CTGGCATATGTATGGCAATTGTT R TATTGACCTGAATCAGCGTTGTCT	664bp	(Graber *etal*., 2007)
bap	F CCCTATATCGAAGGTGTAGAATTGCAC R GCTGTTGAAGTTAATACTGTACCTG	971bp	(Cucarella *etal*., 2004)
smr	F ATAAGTACTGAAGTTATTGGAAGT R TTCCGAAAATGTTTAACGAAACTA	286bp	(Bjorland *etal*., 2001)

Table (2): Cycling conditions for the detection of target genes

Gene	Initial Denaturation	Denaturation	Annealing	Extention	Final Extention	Cycles
nuc	94˚C 5min	94˚C 30sec	57˚C 40sec	72˚C 1min	72˚C 10min	35
bap	94˚C 5min	94˚C 30sec	57˚C 30sec	72˚C 45sec	72˚C 10min	35
smr	94˚C 5min	94˚C 30sec	54˚C 30sec	72˚C 75sec	72˚C 10min	35

2.3. DNA sequencing and phylogenetic tree building

The GeneJET Gel Extraction Kit (K0691, Thermo Fisher, USA) was used to purify three positive PCR products from each *S. aureus bap* and *smr* gene. The sequences were then run by Macrogen Company (Korea). Two-way sequencing using the specific primers used in PCR served as a confirmation of the data's accuracy. The programs Bio Edit 7.0.4.1 and MUSCLE (Multiple Sequence Alignment) (https://www.ebi.ac.uk/tools/msa/muscle) were used to examine the nucleotide sequences acquired in this work. Using the neighbor-joining technique of the aligned sequences deposited in the application CLC genomic workbench 6, the obtained sequences were aligned with reference sequences genes. Six nucleotide sequences for *S. aureus* isolates were produced and deposited in GenBank.

3. RESULTS

3.1. Molecular identification of *S. aureus*

All twelve *S. aureus* isolates were confirmed as *S. aureus* using PCR for *the nuc* gene (12/12) (100%), as shown in Figure (1) and Table (3). Only 10 out of the 12 *S. aureus* isolates were positive in PCR (10/12) (83.33%) targeting *the bap* gene responsible for biofilm formation, as shown in Figure (2) and Table (3). Samples in lane seven and lane 9 were negative (nasal and teat swabs of buffalo, respectively). All 12 *S.aureus* isolates were positive in PCR (12/12)100% targeting *the smr* gene responsible for resistance to antiseptics, as shown in Figure (3) and Table (3).

Table (3): The occurrence of *nuc*, *bap* and *smr* genes in 12 representative *S. aureus* isolates.

Sample	*nuc*	*Bap*	*Smr*
Cow milk	+	+	+
Cow milk	+	+	+
Nasal swab from cow	+	+	+
Nasal swab from buffaloes	+	-	+
Teat swab from cow	+	+	+
Teat swab from buffalo	+	-	+
Dairy utensil	+	+	+
Dairy utensil	+	+	+
Human hand swab	+	+	+
Human hand swab	+	+	+
Human nasal swab	+	+	+
Human nasal swab	+	+	+

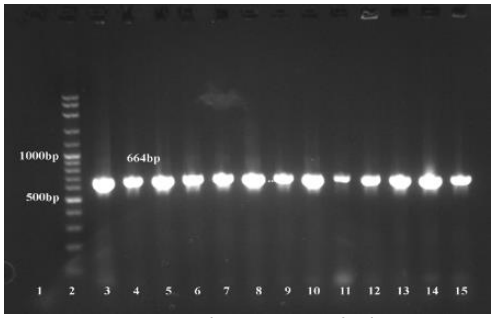

Fig.1. Agarose gel electrophoresis of PCR product amplified from *S.aureus nuc* gene (664 bp). Lane 1 (negative control), Lane 2 (1000 bp DNA Ladder) , Lane 3 (positive control) Lanes 4-15 (representative positive samples) according to previously table respectively.

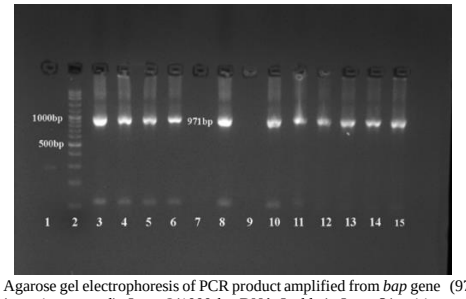

Fig.2. Agarose gel electrophoresis of PCR product amplified from *bap* gene (971bp). Lan1 (negative control), Lane 2(1000 bp DNA Ladder), Lane 3(positive control), Lanes 4-15 were representative samples (lanes 4,5,6,8,10,11,12,13,14,15 are positive while lane7 and 9 are negative) according to previously table respectively.

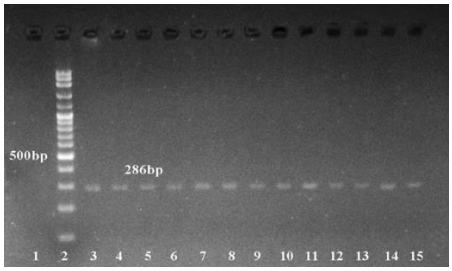

Fig.3. Agarose gel electrophoresis of PCR product amplified from *S.aureus smr* gene (286bp). Lan1 (negative control), Lane 2(1000 b pDNA Ladder), Lane 3 (positive control), Lanes 4-15(representative positive samples) according to previously table respectively.

3.2. DNA sequencing and Phylogenetic analysis

Six nucleotide sequences for *S.aureus* isolates were entered into GenBank using the accession codes OR344353 to OR344355 for the *bap* gene and OR344356 to OR344358 for the *smr* gene (tables 4 and 5), respectively.

Targeting the *bap* gene, the phylogenetic tree formed four clades where all the *S. aureus* isolates recovered from cattle milk (OR344353) and dairy utensils (OR344354) showed a high degree of homology (74%) with *S. epidermidis* isolated from human nosocomial infections (EU011247) in Brazil and low homology (47%) with *S. aureus* isolate from bovine milk (JX403946) in India, as shown in Figure (4).

S. aureus isolate (OR344355) from human nasal swab in Egypt showed a high homology with *S. aureus* isolates (MF278359 (88%) and MF278360 (78%)) isolated from bovine milk in India as shown in Figure (4).

Comparative alignment of the three translated *bap* gene sequences showed typical amino acid sequences of *S.aureus* isolates from bovine milk and dairy utensils (OR344353 &OR344354) (100%) while the human isolate (OR344355) showed major mutations through change and insertion (addition) for example almost along the DNA sequence especially from nucleotide 53 to nucleotide 68 as showed in Figure (5).

Concerning the *smr* gene, all the *S. aureus* isolates from the study (OR344356- OR344358) showed a high homology (43%) with *S. epidermidis* isolated from ovine milk (MK933771) in Italy Figure (6).

Interestingly, *S. aureus* isolated from human clinical samples (AY960707, JF817390, DQ013262) and *S. haemolyticus* (MW296867) isolated from bovine milk formed other clades Figure, (6). Comparative alignment

was recorded for the three translated *smr* gene sequences, which showed almost typical amino acid sequences of *S.aureus* isolates from bovine milk and dairy utensils (OR344356 and OR344357) while mutation by change and addition in human isolates (OR344358) especially from nucleotide 1 to nucleotide 90, almost along the DNA sequences shown in Figure (7).

Table (4): *Bap* gene sequences from GenBank used for phylogentic tree construction

Access No	Species	Host	Sample	Country
OR344353	*S. aureus* (In this study)	Cattle	Milk	Egypt
OR344354	*S. aureus* (In this study)	Cattle	Dairy utensils	Egypt
OR344355	*S. aureus* (In this study)	Human	nasal swab	Egypt
MF278359	*S. aureus*	Bovine	Milk	India
MF278360	*S. aureus*	Bovine	Milk	India
KF972123	*S.epidermidis*	Feline	Conjunctival swab	Poland
JX403946	*S. aureus*	Bovine	Milk	India
OP491171	*S.epidermidis*	Bovine	Milk	India
KF972124	*S.epidermidis*	Feline	Conjunctival swab	Poland
EU011247	*S.epidermidis*	Human	nosocomial infections	Brazil

Table (5): *Smr* gene sequences from GenBank used for phylogentic tree construction

Access No	Species	Host	Sample	Country
OR344356	*S. aureus* (in this study)	Cattle	Milk	Egypt
OR344357	*S. aureus* (in this study)	Cattle	Dairy utensils	Egypt
OR344358	*S. aureus* (in this study)	Human	nasal swab	Egypt
ON448392	*S.aureus*	Human	Nasal Cavity	China
ON448389	*S.aureus*	Human	Nasal Cavity	China
MK933771	*S. epidermidis*	Sheep	Milk	Italy
MW296867	*S. haemolyticus*	Cattle	Milk	India
MK542001	*S.aureus*	Human	Nasal swab	Malaysia
KP687798	*S.aureus*	Human	Blood	Iran
AY960707	*S.aureus*	Human	Clinical samples	China
JF817390	*S.aureus*	Human	Clinical samples	USA
DQ013262	*S.aureus*	Human	Clinical samples	China
JN043515	*S.aureus*	Human	Blood	Malaysia

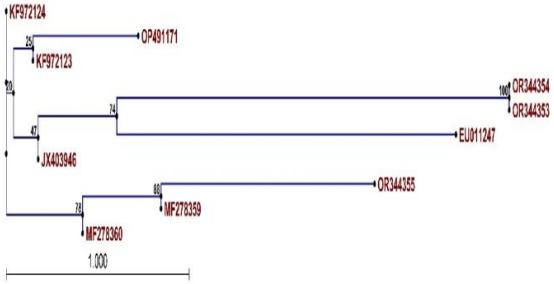

Fig.4. Phylogenetic tree of 3 representative sequences of *S.aureus* isolates *bap* nucleotide sequence (OR344353 - OR344355) and reference sequences, using the neighbor-joining method.

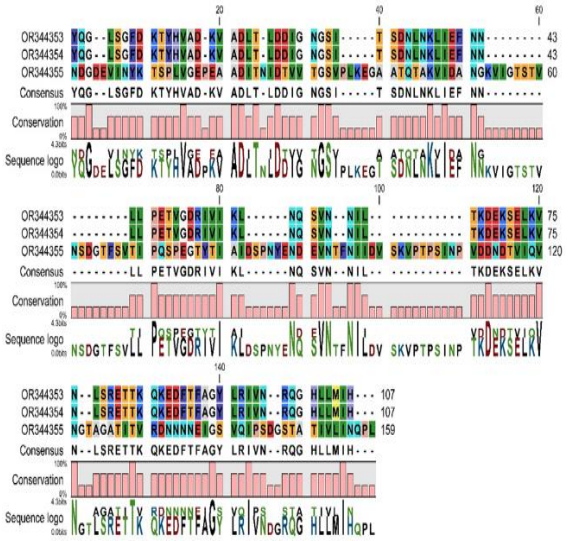

Fig .5. Comparative alignment of the three translated *bap* gene sequences

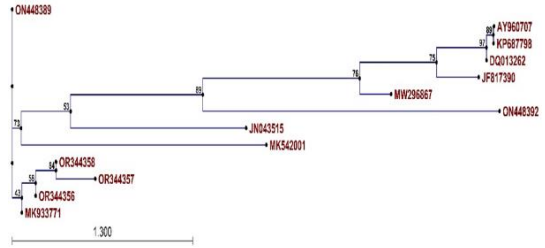

Fig.6. Phylogenetic tree of 3 representative sequences of *S.aureus* isolates *smr* nucleotide sequence (OR344356 -OR344358) and reference sequences, using the neighbor-joining method.

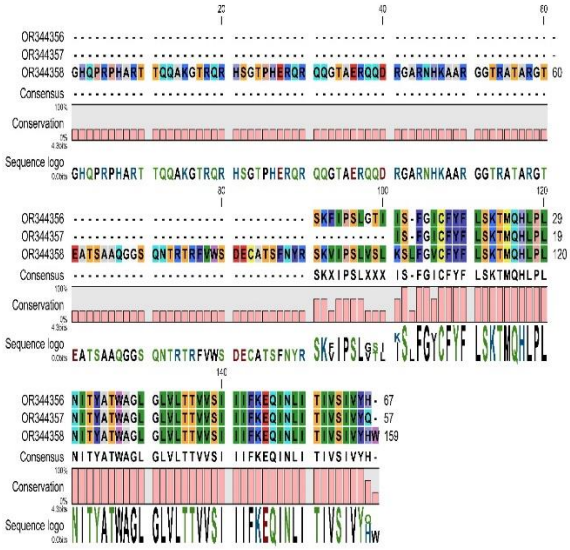

Fig.7.Comparative alignment of the three translated *smr* gene sequences

4. DISCUSSION

Staphylococcus aureus is also known as a significant opportunistic pathogen that affects both humans and animals. It can cause various diseases, including mastitis and food poisoning, by producing heat-stable enterotoxins in food (Phiri *et al*., 2022).

In clinical microbiological diagnosis, *S. aureus* identification is a crucial challenge. The thermonuclear-encoding (*nuc)* gene is frequently used as a particular aim for the detection of *S. aureus* by PCR (Louie *et al*., 2002). So, for *S. aureus* confirmation, we employed the *nuc* gene as a target. This gene, which encodes the thermonuclear, is specific to *S. aureus* only and not found in coagulase-negative staphylococcus spp. In this study, all *S.aureus* isolates (12/12) (100%) were positive for *nuc* gene, and these results were similar to (Islam *et al*., 2019)(100%) and (Rana *et al*., 2018)(100%), however higher than (Ballah *et al*., 2022) who detected (23.81%) of his samples were *S.aureus*. The results of the current study confirm the gene's availability and specificity.

One of the main virulence factors that affect *Staphylococcus aureus's* ability to survive and persist in both the environment and the host is its ability to build biofilm. The most common way that biofilm formation in *S. aureus* is linked to the production of PIA via ica operon-encoded enzymes(Torlak *et al*., 2017) and PIA-independent biofilms, Mediated by the *bap* gene (McCarthy *et al*., 2015).

The *bap* gene was found in this study in(10/12) (83.33%) of *S.aureus* isolates; these results were nearly similar to those (Munive Nuñez et al., 2023)(78.9%) but higher than those (Ballah *et al.*, 2022) (10 %) and (Ibrahim *et al*., 2022) (0%) and lower than (Salimena *et al*., 2016), who reported that (95.6 %) of the isolates have the *bap* gene.

In this study, samples in lane seven and lane 9 were negative for *bap* (nasal and teat swab of buffalo, respectively). These results may be attributed to that buffaloes have a stronger innate immunity to fight against infection (Chanu *et al*., 2018), (Vink, 1995) reported that buffaloes appear to have higher mastitis resistance than cows, and this leads to exposed cows to treatment many times than buffaloes, so *S.aureus* that infect cows increased their virulence capacity especially the biofilm production to resist the antibiotics, so cows produce the *bap* gene higher than buffaloes.

Some studies reported that the *ica* locus is found in the majority of clinical isolates, causing infections in both humans and animals, but *the bap* gene has only been discovered in cattle strains (Vautoretal., 2008), and the absence of

The *bap* gene, according to Vautor *et al*. (2009), implies that the *ica*-dependent pathway is predominately responsible for adhesion and biofilm production in the strains, so *bap-positive* strains are often influential biofilm producers, even in lack of *ica* locus, and can result in severe infections than *bap*-negatives (Lasa and Penadés, 2006).

It has been beneficial for creating effective disinfection techniques to assess the potential resistance of Staphylococci isolates to *SMR* (Qu *et al*., 2019).

In this study, *smr* was present in all *S. aureus* isolates (12/12) (100%), nearly similar to (Liu *et al*., 2015), who reported (77.4%) of *S.aureus* isolates have *smr*, and higher than (Sultan *et al*., 2022 and Suma *et al*., 2023) (7.7 % and 8.33%) respectively.

Bap has been demonstrated to be required to establish biofilm in some staphylococcal strains that cause infections in animals. The *bap* gene was discovered for the first time in a strain of *S. aureus* associated with cow mastitis (Cucarella *et al*., 2001). It was subsequently detected in (CoNS) and associated with animal and human infections (Tormo *et al*., 2005and Latasa *et al*., 2006).

Targeting *bap* gene, the phylogenetic tree formed four clades and all *S.aureus* isolates isolated from cattle milk (OR344353) and dairy utensils (OR344354) displayed high degree of homology with *S.epidermidis* isolated from human nosocomial infections (EU011247) in Brazil and low homology with that of *S. aureus* isolated from bovine milk (JX403946) in India, as shown in (Fig, 4). This can be explained based on the fact that *bap* was first discovered in *S. aureus* isolates associated with bovine mastitis; Bhp, or *bap* homolog protein, is a protein that is similar to *bap* and can be found in human strains of *S.epidermidis*. It may have a similar purpose to *bap* (Tormo *et al*., 2005).

An *S. aureus* isolate (OR344355) from a human nasal swab in Egypt showed high homology with *S. aureus* isolates (MF278359 & MF278360) isolated from bovine milk in India (Fig. 4). This indicated the possibility of transfer of *bap* gene between human and animal *Staphylococcus Spp*. and shows the zoonotic importance of not only *S. aureus* but also CoNS especially *S. epidermidis*.

The spread of resistance genes between staphylococcal species is most likely aided by the production of massive multi-resistance plasmids and their subsequent interspecies interchange (Anthonisen *et al*.,2002).

Concerning the *smr* gene, all the *S. aureus* isolates from the study (OR344356- OR344358) showed a high homology with *S. epidermidis* isolated from ovine milk (MK933771) in Italy (Fig, 6).

These findings support the belief that CoNS serve as reservoirs for various environmental persistence factors, including genes encoding antibiotic resistance, biofilm formation, and multidrug efflux pumps such as *smr* genes. Despite having a lesser pathogenic potential than *S. aureus*, CoNS could actively contribute to the maintenance of these genes in the dairy environment, ready to be transmitted to other bacterial species, as reported by Turchi *et al*. (2020)

The broad distribution of staphylococci containing smr resistance genes in dairy cattle herds appears to be the result of both intra- and interspecies propagation of Qac resistance plasmids and clonal expansion of Qac-resistant strains as confirmed by Bjorland *et al*. (2005).

5. CONCLUSIONS

The current investigation demonstrated that all *S. aureus* isolates harboring *the bap* gene were firm adherent and biofilm producers, in addition to the production of *the smr* gene, which indicates the spreading of Qac-resistant strains. The sequencing and phylogenetic analysis showed that the six translated *bap* and *smr* gene sequences have an almost typical amino acid sequence of *S. aureus* isolates from bovine milk and dairy utensils.

In contrast, the human isolate showed major mutations through change and addition. *S. aureus* isolates showed homology with CoNS and confirmed that CoNS are reservoirs for various environmental persistence factors, including genes encoding antibiotic resistance, biofilm formation, and multidrug efflux pump genes. In conclusion, Dairy workers must wear masks and gloves, follow best management practices when disinfecting, and use the right amounts of disinfectants to ensure that microorganisms are killed and the farms remain hygienic.

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