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Vol. 11(4), pp. 132-140, 28 January, 2017
DOI: 10.5897/AJMR2016.8396
Article Number: 6D164BF62498
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

*Full Length Research Paper*

# Identification and production of biofilm by *Staphylococcus aureus* isolated from buffalo milk and milking environment

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Received 27 November, 2016; Accepted 5 January, 2017

**The interest in production of buffalo milk is increasing in Brazil due to its physico-chemical and nutritional characteristics. However, just as sheep, goats and cows are susceptible to mastitis, so are buffaloes, which is mainly caused by *Staphylococcus aureus*. In this regard, biofilm formation, the ability to escape host immune defense and virulence factor, is presumably a key factor for acute and chronic intra-mammary infection in buffaloes. In this study, biofilm forming capabilities and virulence genes were evaluated in *S. aureus* isolated from buffalo milk and milking environments, using phenotypic and genotypic assays. Thirty two *S. aureus* strains isolated from buffalo milk, milking machines and milkers' hands were obtained from a farm in Analândia, São Paulo State, Brazil. Samples were collected in April, June, October and November 2013. These strains were tested for the presence of *sa*442, *icaA, icaD, clfA, clfB, sarA* and *hla* genes, slime production using Congo Red Agar (CRA), and biofilm formation using microtiter assay. All samples of *S. aureus* were positive for CRA and microtiter assay. Although, *ica*A and *ica*D genes were simultaneously detected in nine of the 32 samples, none of the samples were positive for *ica*A; only seven were positive for *ica*D gene. This suggests that other factors may be involved in biofilm formation. Seventeen strains of *S. aureus* were positive for *sarA* gene, nine for *clfA* gene, 16 for *clfB* and 16 for *hla.* A great variability in *SmaI* restriction profiles of *S. aureus* strains was observed. Thirty isolates were typified and two strains were not by *SmaI* restriction. In addition, 18 pulse types were detected. It is hypothesized that biofilm can be produced by the expression of *ica*D gene only. Our findings suggest that *S. aureus* strains from buffalo milk and milking environment are similar, which contradict the findings obtained from bovine strains. This behavior may contribute to the persistence of mastitis in buffalo caused by *S. aureus*, which results in a potential zoonotic problem. Our results may bring new insights into the development of novel strategies for the prevention and treatment of bubaline mastitis.**

**Key words:** *Staphylococcus aureus*, buffalo milk, biofilm, milking machine, milkers' hands, utensils, mastitis.

## INTRODUCTION

The production of buffalo milk and its derivatives has been growing significantly in Brazil because, according to

Andrade et al. (2011), buffalo milk has more solids, higher concentration of fat, protein and minerals, to produce milk derivatives. However, like cows, buffalos and the most frequently isolated bacteria causing it are *Staphylococcus* spp. and *Streptococcus* spp. (Bastos and Birgel, 2011; Medeiros et al., 2011a).

In bovine mastitis, caused by *S. aureus* and other Staphylococci, the ability to produce biofilm, defined as communities of microorganisms attached to a surface (Martino, 2016), is the most important factor related to difficulties in eradicating acute and chronic intra-mammary gland infections (Szweda et al., 2012). Production of slime enables adhesion of bacteria to the epithelium cells, and persistence in the host tissue, by protecting them from the host immune defense (Szweda et al., 2012). In addition, biofilm may lead to reduced susceptibility to antimicrobial by protecting pathogens from antimicrobial agents (Szweda et al., 2012). Biofilm production by *Staphylococcus aureus* requires intercellular adhesion (*ica*) locus, consisting of *ica*ADB and C genes,which encode the proteins that mediate the synthesis of polysaccharide intercellular adhesin (PIA) (Cramton et al., 1999), and strains that are harboring the *ica*ADBC cluster could potentially become biofilm producers.

The initial attachment of *S. aureus* to epithelial cells of the teat canal depends on the interaction of bacterial surface proteins, such as clumping factors A and B, with host fibrinogen and fibronectin proteins located in the basement membrane, around myoepithelial cells and fibroblasts (Stutz et al., 2011). In addition, *S. aureus clf*A and -B and *Fnb*A and –B genes that encode surfaces proteins are involved in the evasion of host immune responses (Stutz et al., 2011). It has been noted that surface proteins produced by *clf*A gene binds and activates the complement regulator factor I, ClfA and -B and FnbA and -B proteins are involved in the evasion of host immune system (Stutz et al., 2011). Also, other *S. aureus* surface proteins involved in host immune evasion are protein A encoded by *spa* gene (Visai et al., 2009). The immunoglobulin G (IgG)-binding *spA* protein has the capacity to coat *S. aureus* cell surface with incorrectly oriented IgG molecules, thereby preventing phagocytosis as well as classical complement pathway fixation (Atkins et al., 2008).

The adhesion, invasion and ability to induce apoptosis depend on the bacterial growth phases, which are regulated through accessory gene regulator (*agr*) locus and the staphylococcal accessory regulator A (sarA) protein family. S*ar*A gene up regulates the expression of *clf*B and *fnb*A and -B during the exponential growth phase, whereas *agr* down regulates spa and *fnb*A and –B expression in the post exponential growth phase (Cheung et al., 2004). Alpha hemolysin (*hla*) also serves as a virulence factor in *S. aureus*-induced mastitis (Takeuchi et al., 2001).

Factors associated with the transmission of pathogens, such as *S. aureus*, may be related to the milking environment, including milking machine, milkers and procedures related to teat antisepsis before and after milking (Medeiros et al., 2011b). Molecular typing technique used to understand how these pathogens are disseminated is an important epidemiological tool, which may help in establishing an efficient control strategy and hygiene method in milk production. For this type of study pulsed-field gel electrophoresis (PFGE) (Bannerman et al., 1995) is commonly used, and considered the gold standard. This technique is highly discriminatory for detecting genetic variations lower among epidemic strains (Maslow and Mulligan, 1996).

Several reports concerning the production of biofilm by *S. aureus* strains causing mastitis, isolated from different regions of the world, have recently been presented (Vasudevan et al., 2003; Ciftci et al., 2009; Dhanawade et al., 2010). In Brazil, however, the ability of biofilm production by *S. aureus* causing mastitis in buffalo has not been thoroughly investigated.

This study aimed to analyze the ability of *S. aureus* strains isolated from buffalo milk and milking environment to produce biofilm, and detect the presence of virulence genes, using phenotypic and genotypic methods and to investigate the different genomic patterns by PFGE technical.

## MATERIALS AND METHODS

### Collecting samples

In total, 320 milk samples were obtained from female buffaloes from a dairy farm located in Analândia, São Paulo State, Brazil. The samples were collected in April, June, October and November of 2013. After the mammary gland physical examination (Radostits et al., 2007), the milk from each mammary quarter was submitted to the strip cup test and California Mastitis Test (CMT) (Schalm and Noorlander, 1957). It was aseptically cleaned with 70% alcohol. This study was approved by the Ethics Committee on Animal Use (CEUA) of São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal protocol number 013737/13. Sixteen hand samples from consenting milkers, and 32 samples from milking machines were collected with sterile swabs (Pro-Lab Diagnostics), previously stored in peptone water, according to Silva et al. (2000). In addition, eight water samples from the two water hoses used to clean the teats were collected in sterile plastic bottles.

### Microbiological isolation

Isolation and identification of *S. aureus* were performed according

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**Table 1**. Primers' sequence used in this study.

| Genes | Sequence of primers | Size (bp) | References |
|---|---|---|---|
| Sa442 F<br>Sa442 R | 5'-AATCTTTGTCGGTACACGATATTCTTCACG-3'<br>5'- CGTAATGAGATTTCAGTAGATAATACAACA -3' | 108 | Martineau et al. (1998) |
| icaA F<br>icaA R | 5'-CCTAACTAACGAAAGGTAG-3'<br>5'-AAGATATAGCGATAAGTGC-3' | 1315 | Vasudevan et al. (2003) |
| icaD F<br>icaD R | 5'-AAACGTAAGAGAGGTGG-3'<br>5'- GGCAATATGATGAAGATAC-3' | 381 | Vasudevan et al. (2003) |
| clfA F<br>clfA R | 5'-ATTGGCGTGGCTTCAGTGCT-3'<br>5'-CGTTTCTTCCCACGTAGTTGCATTTG-3' | 292 | Yeon-Soo-Seo et al. (2008) |
| clfB F<br>clfB R | 5'-ACATCAGTAATAGTAGGGGGCAAC-3'<br>5'-TTCGCACTGTTTGTGTTTGCAC-3' | 205 | Eidhin et al. (1998) |
| hla F<br>hla R | 5'-AGAAAATGGCATGCACAAAAA-3'<br>5'-TGTTGCGAAGTCTGGTGAAAA-3' | 600 | Wolz et al. (2000) |
| sarA F<br>sarA R | 5'- TTGCGCTAAATCGTTTCATTATTAA-3'<br>5'-AATTTCGTTGTTTGCTTCAGTGA-3' | 275 | Wolz et al. (2000) |

to Lancette and Bennett (2001). Each sample was plated onto Baird Parker agar (Oxoid Ltd., Hampshire, UK), supplemented with egg yolk and tellurite emulsion (1%; Oxoid Ltd.). They were incubated at 37°C for 48 h. Suggestive colonies were gray to black (potassium tellurite reaction) surrounded by clear zones (egg yolk reaction; Capurro et al., 2010). Suggestive colonies were submitted to Gram staining, catalase reaction, clotting of rabbit plasma, acetoin production and presence of clumping factor and protein A (Staphyclin test; Laborclin, Pinhais, Brazil).

**Congo red Agar (CRA) test**

For *S. aureus*, CRA was composed of 37 g/l of brain heart infusion agar (BHI Agar HiMedia Laboratories,), 50 g/l of sucrose (Sigma, St. Louis, MO), and 0.8 g/l of Congo red (Sigma). The morphology of the colonies and their phenotypic changes were studied using CRA cultures as described by Freeman et al. (1989). Plates with Congo red medium were incubated aerobically for 24 h at 37°C to obtain single bacterial colonies. CRA-positive strains appeared as black colonies, while CRA-negative strains remained red.

**Microtiter assay**

Biofilm production was detected using microtiter assay as described by Cucarella et al. (2001), with modifications. Briefly, *S. aureus* strains were inoculated overnight in Tryptic Soy Broth (TSB - Himedia), then the cultures were diluted in 1:200 in TSB with 0.25% glucose (SIGMA). They were incubated overnight in microtiter 96-well polystyrene (Costar 3599, Corning, Tissue Culture-Treated; Corning Inc., Corning, NY), with shaking at 37°C. Next, the wells were washed twice with sterile PBS (pH 7.2). Subsequently, the plates were fixed with methanol and dried. The cultures were stained with 200 µl of 1% crystal violet, per well, for 10 min. Excess crystal violet was removed by gently washing the plate twice with distilled water. Finally, 200 µl of acetic acid was added per well, and the optical density was measured at 570 nm. A well with sterile TSB served as negative control and for positive control was used *S. aureus* strain ATCC 25923, whereby their ODs were subtracted from that of the experimental strains. The mean OD 570 nm value was determined using four replicates, and was considered to be adherence positive at OD 570 nm greater or equal than 0.1 and adherence negative at OD 570 nm less than 0.1 (Christensen et al., 1985).

**DNA extraction**

Bacterial DNA was extracted using the RTP® Bacteria DNA Mini kit (Invitek, Berling, Germany) according to the manufacturer's instructions.

***sa442* gene typing**

The molecular confirmation about *S. aureus* strains was performed by specific primers and protocol adapted described by Martineau et al. (1998). PCR reactions consisted of 20 mM Buffer 1X containing 50 mM magnesium chloride, 100 mM dNTP, 0.5 U Taq Polimerase (Invitrogen, Brazil), 1 µM of primers (Table 1) and 50 ng of termoplate DNA in a total volume of 20 µl. PCR conditions were performed as follows: 94°C for 3 min; 30 cycles each of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. PCR products were subjected to agarose gel electrophoresis. For positive control was used *S. aureus* strain ATCC 25923.

***IcaA* and *IcaD* gene typing**

To amplify the *ica* loci, primers and protocols described by Vasudevan et al. (2003) were used with some modifications. PCR reactions consisted of 20 mM Buffer 1X, 50 mM magnesium chloride, 100 mM dNTP, 0.5 U Taq Polimerase (Invitrogen, Brazil), 1 µM of primers (Table 1) and 50 ng of DNAtemplate in a total volume of 20 µl. PCR cycle conditions were 94°C for 3 min; 30 cycles each of 94°C for 45 s, for icaA gene 58°C for 45 s and icaD

gene 49°C for 45 s, 72°C for 1 min; final extension at 72°C for 7 min.

#### *sarA* and *hla* gene typing

To amplify *sar*A and *hla* gene, primers described by Wolz et al. (2000) were used. PCR reactions consisted of 20 mM Buffer 1X, 50 mM magnesium chloride, 100 mM dNTP, 0.5 U Taq Polimerase (Invitrogen, Brazil), 1 µM of primers (Table 1) and 50 ng of template DNA in a total volume of 20 µl. PCR conditions were 94°C for 3 min; 30 cycles each of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min; final extension at 72°C for 7 min.

#### *clfA* and *clfB* typing

To amplify the products *clf*A and *clf*B genes, primers described by Seo et al. (2008) and Eidhin et al. (1998), respectively were used. PCR reactions consisted of 20 mM Buffer 1X, 50 mM magnesium chloride, 100 mM dNTP, 0.5 U Taq Polimerase (Invitrogen, Brazil), 1 µM primers (Table 1) and 50 ng of template DNA in a total volume of 20 µl. PCR conditions were 94°C for 3 min; 30 cycles each of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; final extension at 72°C for 7 min.

*S. aureus* strain ATCC 25923 and *Streptococcus agalactiae* ATCC 12386 were used as positive and negative control for all PCR. All PCR products were subjected to agarose gel electrophoresis.

#### Pulsed-field gel electrophoresis (PFGE) pulsotyping of *S. aureus* strains

Bacterial DNA for pulsed-field gel electrophoresis (PFGE) typing was digested with SmaI and Pulsed-field gel electrophoresis (PFGE) was conduct according to the protocol established by the Centers for Disease Control and Prevention (CDC, 2001) for *S. aureus* molecular typing. The relatedness of the fingerprints was assessed by using visual examination according to the criteria described by Hiramatsu et al. (1997), computer analysis with BioNumerics software (version 7.1; Applied Maths, Kortrijk, Belgium) with Dice coefficients and clustering by the unweighted-pair group method with arithmetic means. The position tolerance was set at 1.0, and the cluster cutoff was set at an 80% similarity level.

## RESULTS AND DISCUSSION

### *Staphylococcus* spp. and *S. aureus* isolation from milk and milk environment

From the 320 samples of milk obtained, 52 (16.25%) were positive for the California Mastists Test and 268 (83.75%) were negative. From 147 suspected strains of *Staphylococcus* spp. obtained in Baird-Parker Culture Medium and Gram, 26 were confirmed as *S. aureus* by biochemical (Coagulases, Voges Proskauer, Anaerobic Fermentation of Mannitol and Latex agglutination test) and molecular (*sa*442 gene detection) analysis. From the 64 samples obtained from the milking machines, 10 contained *Staphylococcus* spp., with only two being confirmed as *S. aureus.* Of the 16 samples obtained from milkers' hands, four contained *S. aureus*. No strain of *Staphylococcus* spp. was obtained from the eight samples of water from the water hose. The 21.2% of frequency of *S. aureus* in milk samples was lower than the 25% previously reported by Suelam et al. (2012) and Tahoun (2009) with 25 and 78%, respectively. Although, the isolation rate of *S. aureus* in materials such as milking machines (3.12%) was lower when compared with those reported by Suelam et al. (2012), these materials are potential reservoir for Staphylococci, which can be a potential public health hazard. According to Suelam et al. (2012), *S. aureus* could survive at least 35 days in polyethylene, glass, stainless steel and aluminum surfaces due to its ability to attach to hydrophobic material. Detection of 25.0% of *S. aureus* in hand swabs strengthens the potential zoonotic transmission from humans to animals and vice versa. However, this is far below the 36.4% found by Suelam et al. (2012), also from hand swabs. In the study of Lee et al. (2012), *S. aureus* isolates from milkers' hands, utensils, milk from individual cows and bulk tank milk were epidemiologically related. Isolates with the same genetic profile were obtained from insufflators, udder and in milk by Souza et al. (2012). These authors noticed the relevance of milking machines as a transmission mode of mastitis etiological agent. These findings reinforce the need for the correct implementation and practice of milking machine maintenance and milkers' hands hygiene. Suranindyah et al. (2015) observed that improving environmental sanitation with pre milking had significant effects on milk quality.

### Phenotypic and genotypic biofilm production

All samples of *S. aureus* tested for slime production and biofilm production in Congo Red Agar (CRA) were positive, and also all stains were confirmed positive by microtiter assay. Although, other authors reported lower sensitivity and specificity of CRA biofilm detection (Taj et al., 2012; Darwish and Asfour, 2013), and also this test may not be reliable for detection of biofilm (Taj et al., 2012), in this study, this test demonstrated a good correlation with gold standard microtiter assay. Biofilm producer samples were found to be simultaneously positive for the presence of *ica*A and *ica*D genes in nine (28.1%) of the 32 samples. No sample was positive for *ica*A, but seven (21.8%) samples were only positive for *ica*D, suggesting that biofilm can be formed even when only one *ica* loci gene is present.

These results are like those obtained by Darwish and Asfour (2013) with bovine milk samples. These authors observed prevalence rates of *ica*A and *ica*D genes of 15.0 and 62.5%, respectively. Furthermore, Ciftci et al. (2009) found 16 (27.1%) and 38 (64.4%) out of 59 strains were positive for *ica*A and *ica*D genes, respectively. The lower detection of icaA, and therefore the differences in

the prevalence rates can be attributed to the variation in DNA sequences. This may lead to failed amplification of the genes in some isolates and consequently false negative results (Tormo et al., 2005; Ferrer et al., 2012; Darwish and Asfour, 2013). Since *ica* locus is responsible for the production of intracellular adhesion polysaccharide (PIA) (Krewer et al., 2015) and thus forming the multiple layer of cell in a slime matrix characteristic of the staphylococcal biofilm (Darwish and Asfour, 2013), the presence of *ica*A or *ica*D negative strain, with biofilm production in Congo Red Agar and microplate assay isolates, can be accounted for by an *ica* gene independent control of slime production/adhesion mechanism (Liberto et al., 2009).

## Characterization of virulence related genes

In this study, 17 strains of *S. aureus* were positive for *sarA* gene, with nine positive for the *clfA* gene, 16 for the *clfB*, and 16 for the *hla* gene.The *sar*A gene is part of the transcriptional regulatory system, and it is present in the staphylococcal genomes as at least nine major paralogues (Ballal and Manna, 2009). *sar*A is involved in a regulatory system along with *agr* that are involved in the expression of virulence factor. Staphylococcal infection probably involves the synthesis of surface associated proteins, secretion of exotoxins and adhesions. An analysis of the *sar*A mutants suggests that this gene modulates the expression of a number of *S. aureus* genes, including *agr*, *clf*A, *clf*B, *geh*, *hla*, *hlb*, *fnb*A, *fnb*B, *spa*, *ssp*A, *seb*, *sec* and *tst* (Cheung and Zhang, 2002). It remains unclear whether this occurs via a direct interaction between *sar*A and promoter elements of the target gene, or via an indirect route involving some other regulatory factors (Sterba et al., 2003). Similar results were verified in samples of *S. aureus* with *sar*A genes, which also possess *clf*A, *clf*B, or *hla* genes, and in the absence of *sar*A genes, the others were absent as well. Although, these isolates were obtained from samples with negative results in California mastitis test, and they could be considered healthy, or with no indication of subclinical infection, this fact can contribute to the persistence of infection of subclinical staphylococcal infection in animals. Almeida et al. (2013) suggested that the presence of *clf*A gene, a receptor for fibronectin, is involved in the colonization of the mammary gland epithelium despite the clinical picture of the mastitis. In this study, *S. aureus* samples isolated from milkers' hands were positive for *clf*A, *clf*B and *hla* genes, and may be a key factor for the maintenance of these virulence factors in these agents causing disease to both human and buffalo.

The existence of *hla* gene in *S. aureus* isolates are important for these strains, related to staphylococcal infection cases that cause food poisoning (Ariyanti et al., 2011). In this study, it was observed that *hla* genes were distributed among *S. aureus* isolated from buffalo millk, milkers' hands and a milking machine. Detection of these genes in both samples obtained from milk, milkers' hands is important for understanding *S. aureus* epidemiology as *clf*A, *clf*B that encode proteins can promote adhesion of *S. aureus* to a variety of molecules and surfaces, and they have been implicated in cell-cell adhesion (Tsompanidou et al., 2012).

## PFGE profiling

A great variability was observed in the *SmaI* restriction profiles of *S. aureus* strains. Thirty isolates were typified and two strains were not by *SmaI* restriction. Analysis of these fragments presented seven clonal profiles grouped into two different lineages (Figure 1), in which one of them was subdivided into PFGE clonal profile. Comparison of these PFGE clonal profiles demonstrated that clones may have different presence of virulence genes and biofilm forming capabilities in different animals or in different environments (Figure 2). It has been proposed that *S. aureus* isolates from milkers' hands, utensils and milk from individual cows and bulk tank milk were epidemiologically related (Lee et al., 2012). A study by Kot et al. (2012) on 35 *S. aureus* from 18 different herds presented 17 pulse types, while 18 pulse types were detected. On the other hand, Haveri et al. (2008) observed that individual genes or the different gene profiles of multiple *S. aureus* isolates were not linked with the origins of the isolates. This study led to the identification of two main clusters by computer-assisted analysis; clustering of the stains was not based on the host or the pathogenic origin (Figure 2). Strains from milkers' hands and milking machine and milk were clustered together, and two milkers' hands samples matched 100% similarity with samples in the milk, reinforcing the potential zoonotic role of *S. aureus*. Also, the existence of common or similar genotypes among *S. aureus* isolated could reflect a long-term persistence into the bovine mammary gland (Delgado et al., 2011) and maybe buffalo as well. Although, caution would be advised when extrapolating between bovine and buffalo, Zadoks et al. (2002) typed *S. aureus* isolates from intramammary infections, teat skin, teat canals, milking equipment, and milking personnel from 43 bovine herds by using PFGE. These authors also observed that the majority of teat skin isolates were different from those infecting the udders, thus suggesting that specific udder-pathogenic *S. aureus* strains exist, and that they are different from those present on the udder skin. In this study, *S. aureus* strains from buffalo milk and milking environment contradict their findings on bovine strains, but agree with those of Jørgensen et al. (2005) and Haveri et al. (2008). These authors reported that the genotypes of strains from intramammary infection sites and teat skin were indistinguishable by PFGE typing. Only a few previous studies comparing virulence characteristics of *S. aureus* strains isolated from buffalo

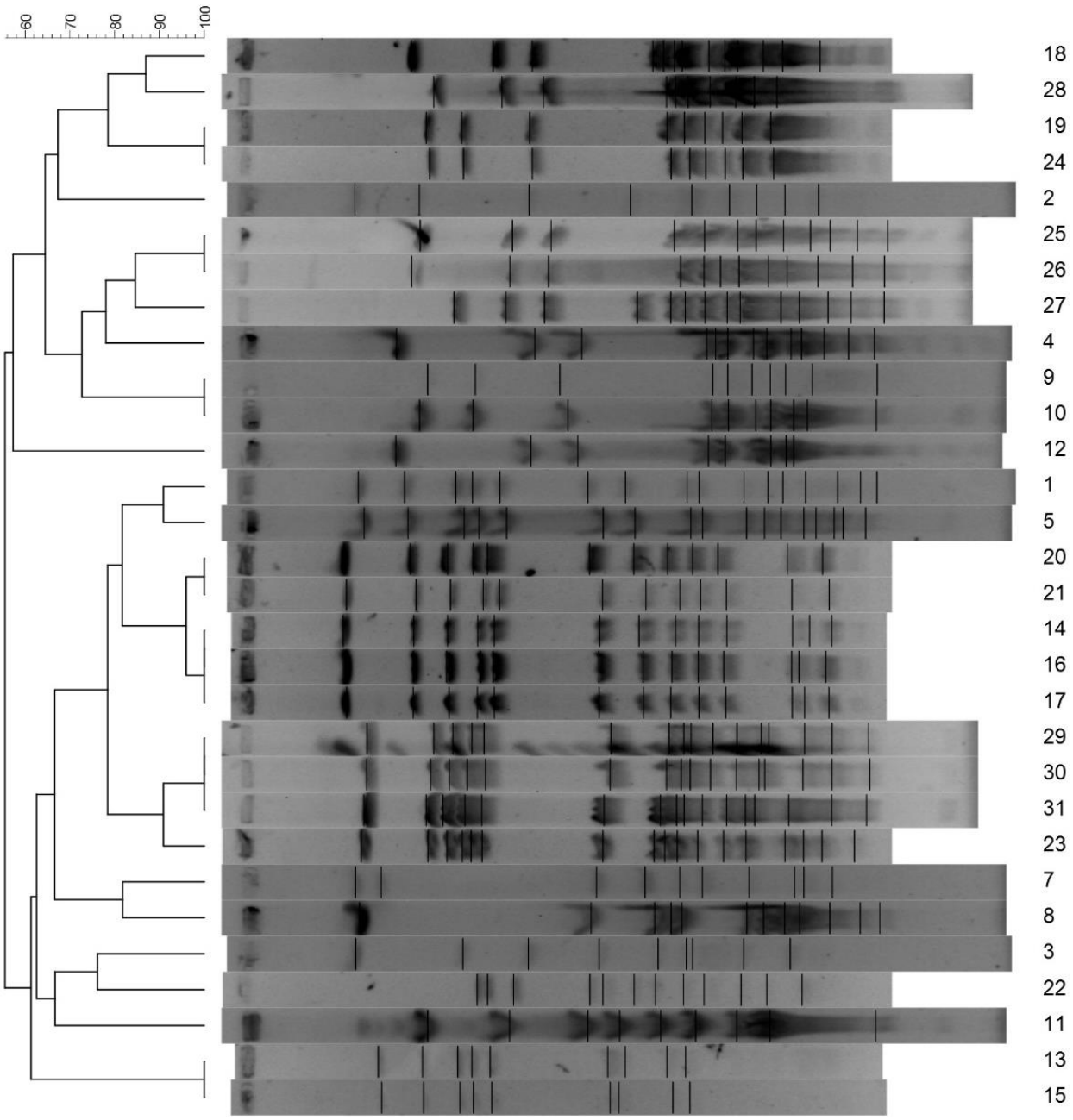


**Figure 1.** Pulsed-field gel electrophoresis (PFGE) of 32 *S. aureus* samples obtained from buffalo milk, milkers' hands and milking machine, digested with SmaI. Clustering were obtained by PFGE analysis in Bionumerics 7.1 software.

milk and extra-mammary sites are available. Fox et al. (2005) found milk-associated *S. aureus* genotypes to be more likely to produce biofilm than genotypes associated with extra-mammary sources.

## Conclusions

Profile evaluation of *S. aureus* strains and its virulence genes characterization related to milkers' hands, milking machine and milk are essential to understand how *S. aureus* can circulate in the milking environment since it is an etiologic and zoonotic agent in buffalo dairy farms. This is because this microorganism harbors virulence genes, even if it is not causing mastitis, it can still offer risk of food poisoning. This strain can spread through the milking environment by milkers' hands, milking machine and milk. This is due to the fact that *S. aureus* strains from different origins were clustered together and shared 100% similarity.

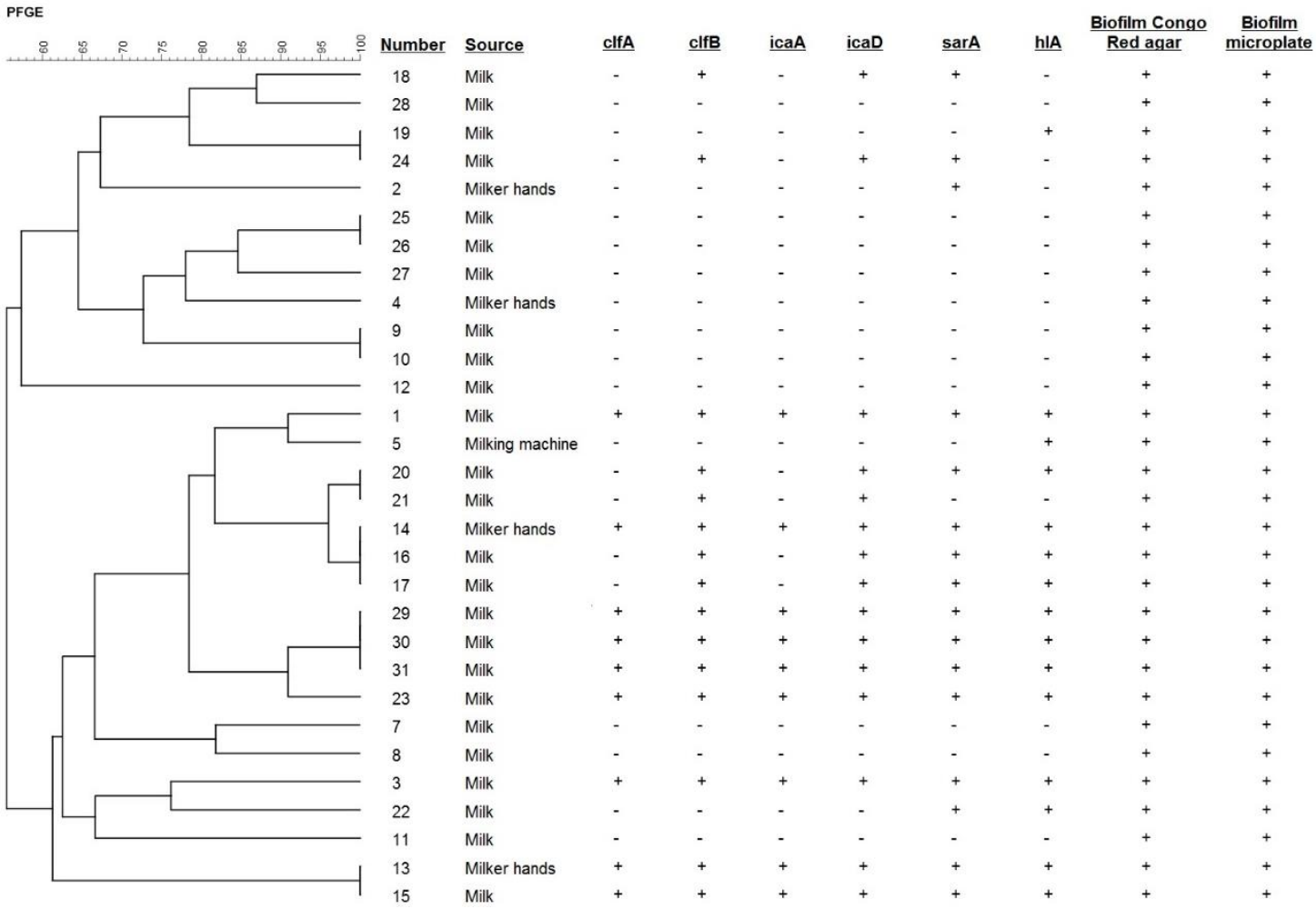


**Figure 2.** Dendrogram based on *S. aureus* pulsotypes detected by PFGE, followed by source of isolation, presence of virulence genes and biofilm formation capabilities on Congo Red Agar and microplate test created by Bionumerics 7.1 software.

The biofilm forming ability of *S. aureus*, the presence of virulence genes and pulsotypes profiling may provide new insights into developing novel biofilm prevention or control strategies for buffalo mastitis and also reinforce the need for correct hygiene protocol for dairy farm to avoid in and out milking site contamination.

## Conflict of Interests

The authors have not declared any conflict of interests.

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