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Full Length Research Paper

Characteristics of 32 *Acinetobacter baumanni* isolates co-harboring OXA-Carbapenemases and 16S rRNA methylase gene

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Multiple drug-resistant strains of Acinetobacter have become common in hospitals worldwide. The problem becomes more acute with existence of hidden Carbapanemase-resistance genes that co-exist with other resistance determinants. Based on this, this research aims to determine the occurrence and co-existence of OXA-type carbapenemases and 16S rRNA methylase gene among clinical isolates of *A. baumannii* in a Jilin hospital, China. Between January 2012 to December 2013, 32 *A. baumanii* non-repetitive strains were isolated from nosocomial clinical specimens. Susceptibility to antibiotics was determined by the standard microdilution method and phenotypic testing was used to detect the presence of carbapenemases. PCR was performed using specific primers for detection of class D carbapenemase genes and 16S rRNA methylase gene. Results revealed that all imipinem resistant *A. baumannii* isolates were resistant to all antibiotics tested except polymycin E. The isolates harbored different Oxacillin Type Carbapenemases, that is, 62.5% of the isolates harbored OXA-51, 37.5% OXA-23, 18.8% OXA-58, 3.1% OXA-24 and 59.4% harbored 16S rRNA methylase gene. Of the 19 isolates harboring 16S rRNA methylase gene, 47.4% coexisted with at least one oxacillinase gene while 40.6% of the 32 *A. baumanii* isolates harbored more than one different oxacillinase gene. At least one oxacillinase gene was detected in 28.1% of the isolates that were phenotypically negative for carbapenemase production. The current study therefore shows co-existence of resistance determinant genes and existence of hidden genes responsible for resistance. Multiple mechanisms are likely to work in synergism to produce this phenotype.

Key words: *A. baumannii*, metallo-β-lactamases, nosocomial infection.

INTRODUCTION

Acinetobacter baumannii is a glucose non-fermentative Gram-negative bacillus classified as an opportunistic pathogen and is usually involved in infectious outbreaks originating in intensive care units (Peleg et al., 2008). The infections caused by *A. baumannii* include bloodstream infections and ventilator-associated pneumonia as well as urinary tract infection. The extensive dissemination of carbapeneme-resistant *A. baumannii* clonal strains

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causing episodes of bacteremia and/or sepsis mostly result of transmission via multiple contaminated surfaces and objects and transiently colonized health care workers' hands (Perez et al., 2007).

The beta-lactam antibiotics are an important group of antibiotics that have been used to treat infections caused by various microorganisms, including *A. baumannii*, (Livermore and Woodford, 2006) due to their efficacy and safety and because their activity can be increased by chemical modification. This was until 1970s' where some isolates of *A. baumannii* were found to be resistant to a wide range of antibiotics including broad spectrum beta-lactams, aminoglycosides and flouroquinolones (Renu et al., 2010). However, decreased susceptibility to carbapenems has been reported worldwide (Uvizl et al., 2011; Trecarichi et al., 2011). This fact is associated with high mortality and morbidity rates, prolonged hospital stays and increased treatment-related costs.

The emergence of carbapenem resistance in *A. baumannii* has become a global concern since these β-lactams are often the only effective treatment left against many multidrug-resistant strains. The major mechanism of carbapenem resistance in *A. baumannii* is the production of OXA-type genes (OXA-23,OXA-24,OXA-51 and OXA-58) and/or metallo-β-lactamase (VIM,IMP,NDM) (MBL) enzymes belonging to Ambler classes D carbapenemases or Class B Metalo-β-lactamase (MBL) respectively (Poirel et al., 2008). A recent development has been the discovery of a novel group of narrow-spectrum OXA β-lactamases in carbapenem-resistant strains, some of which have acquired the ability to hydrolyse the carbapenems (Opazo et al., 2012). These enzymes belong to three unrelated groups of clavulanic acid-resistant □-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid- or chromosomally-encoded. *A. baumannii* also possesses an intrinsic carbapenem-hydrolysing oxacillinase, the expression of which may vary and may play a role in carbapenem resistance (Lee et al., 2006).

These OXA-type cabarpanemaes (OTC) have been described in different provinces of China. Wang et al. (2011) indicated that OXA-58-like, a carbapenem-resistance determinant located on transferable plasmids and spread by clonal dissemination in most cases, is an emerging threat in China. OXA-51 and OXA-23 in China have been reportedly carried by almost all *A. baumannii* strains isolated due to their chromosomal location (Dijkshoorn et al., 1998).

The 16S rRNA methylase gene is often responsible for the phenotype of Gram-negative pathogens that show high MICs to most aminoglycosides partly due to its enzymatic modification and methylation. 16S rRNA methylase gene has been isolated in *A. baumannii* strains from Korea, Japan, China and other parts of the world with the first gene reported in a strain of *Klebsiella pneumoniae* in France (Wang and Chen, 2005).

The 16S rRNA methylase genes are mostly located on transposons within transferable plasmids, which provide them with the potential to spread horizontally. Some of the *A. baumannii* strains have been found to coproduce extended-spectrum β-lactamases or metallo-β-lactamases, contributing to their multidrug-resistant phenotypes. In addition, studies reveal that *A. baumanii* strains harboring more than one OXA-encoding gene are emerging (Brown and Amyes, unpublished results).

Identification of OTC carrying isolates has been a challenge due to emergency of hidden carbapenem susceptible oxacillinases which may be missed in daily Lab practice. These carbapenem susceptible organisms with hidden oxacillinase genes can spread unnoticed in hospitals. Furthermore, these resistance determinants are located on transferable plasmids consequently easily spread.

The objective of this study was to characterize *A. baumannii* isolates carrying Oxa-type carbapenemase (OTC) from clinical specimens collected from patients in a Jilin hospital in order to clarify the phenotypes and genotypes. The study emphasized on determination of the occurrence and co-existence of OXA-type carbapenemases with 16S rRNA methylase gene.

MATERIALS AND METHODS

Bacteria strains

A total of 32 non-repetitive imipenem-resistant isolates of *A. baumannii* were recovered from clinical respiratory infections in hospitalized patients from January 2012 to December 2013 in a Jilin hospital, China. The identification of strains was performed by standard microbiology procedures including growth on MacConkey agar and simple biochemical tests like Oxidase test, growth on TSI and Eosin Methylene Blue Agar and ability to grow at 44°C. Analytical profile index (API) system procedure was used to biochemically identify the strains.

Kirby Bauer disc diffusion

Antibiotic susceptibility testing was done on all isolates using commercially available discs by the Kirby Bauer disc diffusion method and interpreted as recommended by Clinical Laboratory Standards Unit (CLSI, 2010). The turbidity of the culture suspension was diluted to match 0.5 McFarland standards according to CLSI. The following antimicrobial discs were used: ampicillin (10 µg), piperacillin (160 µg), cephelothin (30 µg), tetracycline (30 µg), aztreaonm (30 µg), chloramphenicol (30 µg), polymycin E (10 µg), cefotaxime (30 µg), ceftaxime (30 µg) and ceftriaxone (30 µg).

Determination of MIC

In all *Acinetobacter spp.* isolates, susceptibility to antibiotics was determined by a standard micro-dilution method according to the Clinical Laboratory Standards Unit (CLSI, 2010).

Phenotypic determination of carbapenemase production

Carbapenemase production in *Acinetobacter spp*. Isolates with a

Table 1. Different genes/enzymes responsible for resistance in *Acinetobacter baumannii* (John Roberts Table).

Gene/Enzymes	Class/family	Antimicrobial class
OXA-23, OXA-24, OXA-51, OXA-58	Class D carbapenemases	Carbapenems
VIM,IMP,NDM	Class B Metalo-β-lactamase (MBL)	Carbapenems
16S rRNA		Aminoglycosides

OXA: Oxacillinase.

MIC for meropenem of >2 mg/L was phenotypically determined by the Modified Hodge Test (MHT) for carbapenemases production (Amjad et al., 2011).

PCR detection of OXA- genes and 16S rRNA genes

The boiling method was used to extract DNA from the bacteria as described by Vaneechoutte et al. (1995). Briefly, one colony of a pure culture grown on TSI slanting agar was re-suspended in 50 µl of LB broth and incubated at 37°C in an orbital shaker for 14 h. 50 µl of the growth was centrifuged and resultant pellet re-suspended in 50 µl of distilled water and heated at 100°C for 10 min. After centrifugation in a micro-centrifuge, at 6000 x g for 3 min, the supernatant was kept at -20°C for further use.

PCR was carried out in a 25 µl reaction volumes with 1 µl of extracted DNA, 12.5 µm of the PCR master Mix containing 1.25 U of Taq Polymerase, 1 x PCR buffer containing 0.1 mM $MgCl_2$ and 240 µM of each dNTP. Specific primers used are shown in Table 1. PCR conditions were as follows: 94°C for 5 min and then for 35 cycles, 94°C for 30 s, 57°C for 45 s, 72°C for 30 s and then final extension at 72°C for 10 min. Amplified products from the isolates were analyzed by electrophoresis on 1% (w/v) agarose gel stained with Gel Red.

Competent cells were prepared from protocol given by Joe (2005). The DNA band of interest was excised, purified with a QIAqick PCR purification kit, ligated to pGEM-T Easy vector (Promega), transformed into *E. coli*$_{DH5\alpha}$ competent cells, and the white colonies which were successfully transformed and selected were screened. Nucleotide sequencing was performed directly on cloned fragments using an ABI Prism 377 DNA sequencer as a control and to rule out non-specificity in case of novel gene sequences. Sequence similarity searcher was carried out with the BLAST program available at the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov).

RESULTS

Antimicrobial susceptibility to imipenem determined by Kirby Bauer disc diffusion

Phenotypically, all *A. baumannii* strains were found to be extended-spectrum β-lactamase-producing (ESBL) and all isolates were imipenem resistant (data not shown). In imipenem-resistant *A. baumannii* isolates, phenotyping showed resistance to chloramphenicol (100%), gentamicin (100%), amikacin (100%), ciprofloxacin (100%) and levofloxacin (100%) as well as β-lactams and aztreonam (100%). However, all isolates (100%) were susceptible to polymyxin E.

Minimum inhibitory concentrations (MICs)

The MICs of the 32 *A.baumanii* isolates were determined. All isolates (100%) were resistant to all drugs tested, that is, Cephalosporines (Cefetamet, Ceftazidime, Cefotaxime, Cefuroxime and Ceftriaxone) as their MICs were > 2 mg/L. These isolates were resistant to three or more classes of antibiotics. According to the definition given by A. P Magiorakos et al. (2011), we described these isolates as MDR.

Phenotypic determination of carbapenemase production in *A. baumannii*

Imipenem-resistance by disc diffusion method was found in all 32 isolates of *Acinetobacter spp.* Of the 32 imipenem-resistant *Acinetobacter spp*. 18 (56.3%) were carbapenemase producers when tested by MHT. Whereas, remaining 14 (43.8%) isolates did not show evidence of carbapenemase production.

16SrRNA gene

Thirty two Imipinem resistant *A. baumannii* strains were tested for 16S rRNA methylase genes. Of the 32 *A. baumanii* isolates, 19 (59.4%) harbored 16S rRNA methylase gene of which 11 (61.1%) tested positive for carbapanemase and also 8 (57.1%) out of 14 of the non-carbapenemase producing isolates harbored 16S rRNA methylase gene. We also found that 47.4% of the isolates harboring 16S rRNA methylase gene co-existed at least with one oxacillinase gene (Table 2 and Figure 1).

Carbapenem-resistant genes

Thirty-two imipinem resistant *A. baumannii* strains were

Table 2. Multiplex PCR primers for detecting genes encoding oxa carbapenemase (John Roberts Table).

Primer	Sequences	Expected size (bp)
OXA-23 F	GATGTGTCATAGTATTCGTCGT	501
OXA-23 R	TCACAACAACTAAAAGCACTGT	-
OXA-51 F	TAATGCTTTGATCGGCCTTG	353
OXA-51 R	TCGATTGCACTTCATCTTGG	-
OXA-24 F	ATGAAAAAATTTATACTTCCTATATTCAGC	246
OXA-24 R	TTAAATGATTCCAAGATTTTCTAGC	-
OXA- 58 F	AAGTATTGGGGCTTGTGCTG	599
OXA- 58 R	CCCCTCTGCGCTCTACATAC	-
16SrRNAF	GTCGTAACAAGGTGGTTGC	1450
16SrRNAR	GGGTTTCCCGTTCGGAAAT	-

OXA: Oxacillinase.

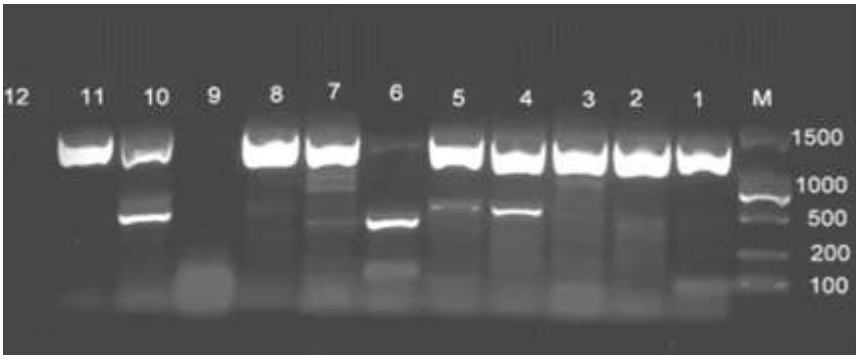

Figure 1. Agarose gel electrophoresis of PCR patterns of genomic DNA from clinical isolates of *A. baumannii* harboring 16srRNA methylase gene. Lane M: 100bp DNA size marker; lane 1~11: are 1450bp positive for 16SrRNA; Lane 6 and 9 are negative for 16SrRNA; Lane 12: negative control.

Table 3. Distribution of 16srRNA metheylase gene among MBL and non MBL producing *Acinetobacter baumannii* (John Roberts Table).

Types	MBL producers	No MBL producers	Total (n =32)
16S rRNA gene	11(61.1%)	7(38.9%)	18(56.25%)
No 16S rRNA gene	8(57.14%)	6(42.86%)	14(43.75%)

MBL: Metallo beta lactamase.

studied to determine presence of oxacillinase genes. Twenty out of 32 (62.5%) *A. baumannii* isolates harbored OXA-51 gene with 75% of these isolates being carpanemase producers and the remaining 25% Non carbapenemase producers. Of the 32 *A. baumannii* strains, 37.5% isolates harbored OXA-23 gene and 75% of them were carbapenemase producers. Six out of 32 (18.6%) *A. baumannii* isolates harbored OXA-58 gene and 50% were non- carbapenemase producers. Only one isolate contained OXA-24 gene.

Co-existence of different bla_{OXA} genes among 32 isolates of *A. baumannii* are indicated in Tables 3 and 4. None of the isolates carrying bla_{OXA-24} associated with bla_{OXA-23} or bla_{OXA-58} genes. A big percentage (72.7%) of *A. baumannii* isolates containing bla_{OXA-51}-like gene coexisted with all other OXA-like genes, because this gene is intrinsic to *A. baumannii* isolates (Figure 2).

Cloning and sequencing confirmed that the PCR products were 100% identical to the OXA-51 genes (GenBank accession nos AY795964 and AY949204).

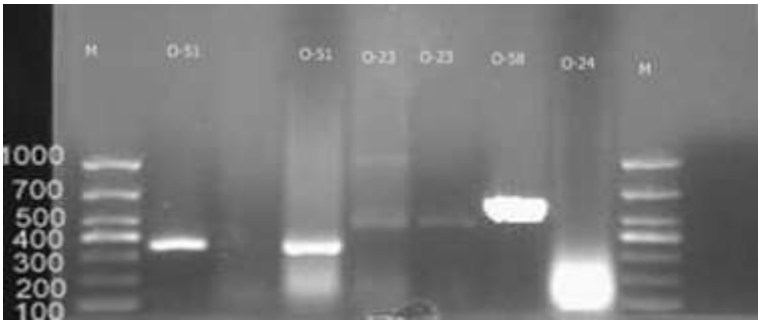

Figure 2. Agarose gel electrophoresis of PCR patterns of genomic DNA from clinical isolates of *A. baumannii* harboring OXA-type Carbapenemase genes. Lane M: 100bp DNA size marker; O-51 is OXA-51, O-23 is OXA-23, O-58 is OXA-58 and O-24 is OXA-24 genes respectively.

Table 4. Distribution and co-existence of different bla_{OXA} genes among clinical isolates of carbapenem resistance *Acinetobacter baumannii* (John Roberts Table).

Isolate	MBL	OXA-51	OXA-23	OXA-24	OXA-58	16S rRNA
1	X	X	X	0	0	X
2	X	X	0	0	0	X
3	X	0	0	0	0	X
4	0	0	0	X	X	X
5	0	0	X	0	0	X
6	0	0	X	0	0	0
7	X	X	0	0	0	X
8	X	X	X	0	0	X
9	X	X	X	0	0	0
10	X	X	0	0	0	X
11	0	0	0	0	X	X
12	0	0	0	0	0	0
13	X	X	X	0	0	0
14	0	0	0	0	0	X
15	X	X	0	0	X	X
16	X	X	0	0	0	X
17	0	X	0	0	0	X
18	0	0	0	0	0	0
19	X	0	0	0	0	0
20	0	0	0	0	X	0
21	X	X	X	0	0	0
22	X	X	0	0	0	0
23	X	X	X	0	0	X
24	0	X	0	0	0	X
25	X	X	0	0	0	X
26	X	0	X	0	X	0
27	0	X	0	0	0	0
28	X	X	X	0	0	0
29	0	X	X	0	X	X
30	0	X	X	0	0	X
31	X	X	0	0	0	0
32	X	X	X	0	0	0
Total	**19**	**20**	**13**	**01**	**06**	**18**

OXA: Oxacillinase; MBL: Metallo beta lactamase. X= Positive, 0 = Negative.

DISCUSSION

Carbapenem resistance in *A. baumannii* has been increasingly detected in Asian countries, which is the same case with China (Zong et al., 2008; Wang and Chen, 2005). In China, national resistance surveillance data from intensive care units (ICU) at 19 teaching hospitals (1996 to 2002) showed that 5% of *Acinetobacter* isolates were resistant to imipenem (Wang and Chen, 2005). However, another national surveillance program involving 10 geographically disparate hospitals found that resistance to carbapenems increased from 4.5% in 2003 to 18.2% in 2004 (Wang and Chen, 2005).

Carbapenems (e.g., imipenem and meropenem) and aminoglycosides are the drugs of choice in the treatment of *Acinetobacter* infections in almost all medical centers, but are being compromised by the emergence of carbapenem-hydrolyzing-lactamase (carbapenemase) of molecular classes D, especially OXA-type and methylasation of 16S rRNA methylase gene respectively. Furthermore, several studies have indicated that imipenem may be hydrolyzed by the extended-spectrum-lactamases of Gram-negative bacteria.

In this study, *A. baumannii* isolates were described as Multi-Drug Resistant (MDR) strains as the isolates were resistant to all antibiotics tested with exception of polymyxin E. This was based on the definition of MDR as given by Magiorakos et al. (2011). These findings also agree with Muthusamy and Boppe's claim that strains of *A. baumannii* are emerging as MRD (Muthusamy and Boppe, 2012). These results agree with the findings of Dalin et al. (2015) who observed that, all the isolates of *A. baumanni* isolated in an affiliated hospital of Jilin, China were resistant to all antibiotics.

By using the modified Hodge test as a screening test for carbapanemase production (Amjad et al., 2011), we found that 19 (56. 25%) of 32 *A. baumannii* isolates were carpanemase producing isolates. This agrees with Irfan et al. (2008) observation suggesting the presence of OTC enzymes since they are considered to be the mechanism of resistance in these organisms. This finding is also consistent with reports from other tertiary care hospitals around the world and even China (Zong et al., 2008; Wang and Chen, 2005). The resistance of *A. baumanni* to antimicrobial agents is mediated by almost all resistance mechanisms found in bacteria. Methylation of 16S rRNA methylase gene has emerged as a mechanism of high-level resistance to aminoglycosides among Gram-negative bacteria. In this study, the 16S rRNA methylase genes were amplified and detected in 19 (59.38%) of the isolates and 61.1% were carbapenemase producers. These findings are consistent with other published reports suggesting that *A. baumannii* carries 16S rRNA methylase gene (Kyungwon et al., 2011). As 16S rRNA methylse gene confer high-level resistance to aminoglycosides, its occurrence in *A. baumannii* limits the clinical use of aminoglycosides.

Our study reveals that 50% of the of 32 *A. baumannii* isolates studied harboring 16S rRNA methylase gene co-existed with at least one oxacillinase genes. This finding agree with that of Strateva et al. (2012) which showed here that *A. baumannii* isolates producing 16S rRNA methylase gene harbored both $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-51}}$-like genes. This is because these genes are linked with mobile genetic elements and usually confirmed as located on large conjugative plasmids, allowing potential spread among bacterial populations.

In addition, this association with other genes encoding resistance to clinically relevant antimicrobials such as ß-lactams (bla_{SHV}, $bla_{\text{CTX-M}}$, plasmid-mediated AmpC), carbapenems ($bla_{\text{SPM-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$) and fluoroquinolones (plasmid-mediated *qepA*, *aac(6')-Ib-cr* and *qnr* family), allows potential co-selection and maintenance of resistance by use of other antimicrobial agents.

Beta-lactamases are the most prevalent group of enzymes that are responsible for resistance in *A. baumanniii* and more than 50 different types have been identified so far in this strain. Several reports from around the world indicate a large increase in the rates of carbapenem-resistant *A. baumannii* from 8% in 2003 to 52% and 74% in 2005 and finally to 96% in 2007 (Mendes et al., 2009) due to these group of hydrolytic enzymes.

As shown in our study, 20 (62. 5%), 13 (40. 63%), 6 (18.75%), and 1 (3.13%) of the 32 multidrug-resistant strains studied carried OXA-51, OXA-23, OXA-58 and OXA-24 genes respectively. Moreover, 10 (31. 25%) isolates were positive for both OXA-51, OXA-23genes. Our data support those of other studies that demonstrated that OXA-51 may be used as a marker to identify *A. baumannii* (Qi et al., 2008). The OXA-23 genes have been documented in strains associated with outbreaks of carbapenem resistant *A. baumannii* in Asia, Europe and South America (Merkier and Centrón, 2006). Four strains were OXA-58 positive and only 1 (3.4%) strain was positive for OXA-24. This is consistent with studies from other hospitals in Jilin and entire China which suggests that the most widespread oxacillinase gene in *A. baumannii* is OXA-51, OXA-23, OXA 58 and OXA-24 in the order (Su-ying et al., 2015). This study also reveals that 9 (28.1%) out of 32 *A. baumanni*i isolates carried at least one oxacillinase gene though they were negative for carbapenemase production. This suggests the existence of hidden carbapenemase genes. These hidden genes can easily be missed in daily laboratory practice hence posing as a diagnostic and therapeutic challenge and can easily spread unnoticed in the hospital. We also found that 13 (40.63%) out of 32 isolates carried more than two oxacillinase genes, and were MDR.

The main limitation of this study is that it was confined to a single centre and it would be valuable to extend the origin of the strains. Another limitation is the small sample

size which led to a lack of power to determine the individual effects of each broad spectrum antibiotics.

Conclusion

The emergence of broad-spectrum antibiotic resistance profiles in *A. baumannii* clinical isolates is worrying in the hospital. The current study shows co-existence of resistance determinant genes and also existence of hidden genes responsible for resistance. Multiple mechanisms are likely to work in synergism to produce this phenotype. The co-existence and the multiple mechanisms working in synergy led us to an insight of defining *A. baumannii* strains in this study as extensive drug resistant *A. baumannii* (XDRAB), as there was still hope with the use of the highly toxic polymyxin E. The results here highlight that enhanced surveillance and health policies for the detection and control of these MDR pathogens are urgently needed to avoid the emergence and spreading of such organism.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Full Length Research Paper

Phytochemical screening of *Azadirachta indica* A. Juss for antimicrobial activity

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***Azadirachta indica* A. Juss, known as neem (Meliacea family), has insecticide and pesticide properties, and many studies have shown their efficacy as antifungal, anti-inflammatory, among others. Studies for the development of drugs from plants are rising due to several factors such as bacterial resistance, indiscriminate use and the adverse reactions of antibiotics. In this study, phytochemical triage and thin layer chromatography analysis were performed, with similar results as the presence of flavonoids, tannins and terpenes. The antimicrobial activity showed that the ethyl acetate extract and butanol fraction presented greater activity against *Streptococcus mutans* and *Streptococcus mitis* presenting a MIC = 50 µg/ml for these strains, and the strain *Enteroccocus faecalis*, the hydroethanolic extract and aqueous fraction were most promising samples with a MIC = 50 µg/ml and MIC = 25 µg/ml, respectively. Therefore, it encourages the continuation of studies, aiming at the development of cosmetics or toothpaste.**

Key words: Antimicrobial activity, *Azadirachta indica*, minimum inhibitory concentration, oral strains, phytochemistry.

INTRODUCTION

The widespread use of a limited number of antimicrobial agents concomitantly with the reduced arsenal of drugs with antimicrobial function, has led to the development of resistance to drugs that oppose both fungal and bacterial infections, which has been an increasing problem (Alexander and Perfect, 1997; Rex and Pfaller, 2002; Zida et al., 2016).

The need to find alternatives for microbial control has induced a lot of research in order to seek products that are effective and economical. Studies of natural products with biological activity offers credible alternatives for microbial control, particularly from bioactive products derived from plants with therapeutic properties of routine use (Albuquerque, 2001). Exotic plants well adapted to the Brazil climate have also shown several properties in traditional medicine, insecticides, among others, as in the

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case of *Azadirachta indica* A. Juss (neem). Originally from Southeast Asia, this plant is also found in tropical and semitropical regions like India, Bangladesh, Pakistan and Nepal. *A. indica* tree belongs to the family Meliaceae (Girish and Bhat, 2008; Alzohairy, 2016; Schmutterer, 2004) and its most important active constituent is azadirachtin (Hossain et al., 2011; Alzohairy, 2016). The most characteristic metabolites of this family are called limonoids, which are tetranortriterpenoides; which has considerable interest due to fascinating structural diversity and its broad biological activity (Tringali, 2001).

Study evidenced that plants fruits, oil, leaves, bark and other parts have important role in diseases prevention due to their rich source of antioxidant (Alzohairy, 2016). Quercetin and sitosterol, polyphenolic flavonoids, were purified from neem fresh leaves and were known to have antibacterial and antifungal properties (Govindachari et al., 1998; Alzohairy, 2016).

There are some available chromatographic methods and among these, the thin layer chromatography is the most widely used for their fast analysis, qualitative detection, and provision of semi-quantitative information of the most active constituent of the drug; thereby enabling an assessment of the drug quality (Wagner et al., 1996). According to previous study (Carneiro et al., 2012), the thin layer chromatography of the fractions hexane, dichloromethane and ethyl acetate showed a predominance of terpenes. The limonoids or tetranortriterpenoides which are very common on leaves and fruits of *A. indica* belong to this class of substances (Shumutterer, 2002; Roy et al., 2006).

The neem has a wide range of several therapeutic properties based on its characteristics, such as antifungal, antibacterial, antioxidant, antiviral, anti-inflammatory, analgesic, antipyretic, and immune stimulant activity (Subapriya and Nagini, 2005; Mustafa, 2016). The leaf extract is commonly used as an antibacterial agent. In addition, the neem has several applications, such as antiseptic, healing, anthelmintic; use in medicinal soaps, creams and toothpaste (Schmutterer, 2004; Dutta and Kundabala, 2005, 2013; Mustafa, 2016).

Dental caries and oral health/dental health are multifactorial diseases related to diet, oral microbiota, hygiene, salivary characteristics, and are inseparable part of general health, which can lead to considerable pain and suffering. It has an impact on a person's speech, selection of food, quality of life, and general well-being. In view of the prevalence of oral diseases, their impact on individuals and society and the expense of treatment, may be considered a major public health problem and they are listed among the most common of the chronic diseases that affect mankind. Oral diseases are the fourth most expensive diseases to treat in certain countries (Chandra Shekar et al., 2015; Sheiham, 2005). *A. indica* was tested as herbal alternatives to endodontic irrigants in comparison with sodium hypochlorite (standard irrigant), and this study showed the zones of inhibition of leaf extracts had antimicrobial properties with significant greater zones of inhibition than 3% sodium hypochlorite (Alzohairy, 2016; Honmode et al., 2013).

According to the World Health Organization (WHO) report, dental caries, though exhibiting a declining trend in many parts of the industrialized world; is still an important public health concern in many developing countries. The statistics suggest that dental caries affect 60 to 90% of school going children in developing countries (Chandra Shekar et al., 2015; Petersen, 2003).

From a microbiological point of view, the appearance of lesions of the disease is linked to the complex structure of the dental biofilm, involving the participation of several micro-organisms, such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus sanguis,* which colonize the surface of the teeth shortly after their outbreak in the oral cavity (Kawashima et al., 2003; Svensater et al., 2003; Lindquist et al., 2004; Marsh, 2004; Seki et al., 2006).

MATERIALS AND METHODS

Collection and preparation of plant material

The collection of *A. indica* A. Juss sheets was held in the Garden of Medicinal Plants and Toxin of the University of Pharmaceutical Sciences of UNESP, Araraquara, situated at geographic coordinates 21°48'52.44"S and 48°12'07.13". It was also collected copy for the preparation of voucher specimen, which was sent to tipping in the Herbarium of São José do Rio Preto under SJRP31236 number.

The extracts were obtained from the leaves, which were washed with sodium hypochlorite (0.2%) and deionized water, and dried in an air circulating oven at 40°C for three days, followed by grinding the dried leaves in knife mill, and then the material was passed at 0.42 mm mesh sieve in order to reduce and standardize the particle size. The hydroethanolic extract was obtained by percolation of *Azadirachta indica* A. Juss. using 70% ethanol as solvent at a flow rate controlled to 10 drops/min until exhaustion of the drug. The volume of the solution extractive obtained was reduced on a rotary evaporator (pressure and temperature) and then, the drying of the residue was completed in petri dishes in an oven at 40°C. The ethyl acetate extract used as extraction liquid was obtained by maceration in an attempt to achieve antimicrobial activity of different compounds extracted by this solvent. In order, 100 g of the plant drug moistened with 200 ml of the solvent was placed, gradually adding more 200 ml. After that, it was put in contact with the heating mantle at 30°C, stirring every 1 h in 1 h for 3 h. The extraction solution was first dried on rotaevaporator, and then dried in an oven at 40°C. Fractions to be tested were obtained by solubilizing up 2.00 g of the dry hydroethanolic extract in 100.00 ml of distilled water, using separator funnel of 300.00 ml. Then the liquid / liquid partition with increasingly polar solvents began (n-hexane, ethyl acetate and n-butanol) in similar proportions of organic solvent and aqueous solution until complete separation of the respective phases (with an average duration 15 min). The obtained fractions were concentrated to dryness in chapel.

Phytochemical screening

To characterize the major groups of secondary metabolites such as

Table 1. Mobile phase and reagents used for determination of secondary metabolites using technique of thin layer chromatography.

Secundary metabolite	Mobile phase	Reagent
Flavonoids	*n*-butanol:acetic acid: water (63:33:4)	NP-PEG+UV
Alkaloids	*n*-butanol:acetic acid: water (70:28:2)	Dragendorff
Terpenes	*n*-hexano:ethyl acetate:isopropanol (70:28:2)	Anisaldehyde sulfuric
Tannins	Ethyl acetate:formic acid:water (90:10:10)	Ethanolic solution of ferric chloride 1%

Table 2. Phytochemical screening of the plant from *Azadirachta indica*.

Secundary metabolite	Results
Saponins	Positive
Tannins	Gelatin 2.5% - Negative; Ferric chloride 1% - Negative
Flavonoids	Positive
Anthraquinone	Negative
Cardiotonics glycosides	Negative
Alkaloids	Negative

tannins, flavonoid, glycoside cardiotonics, saponins, anthraquinones and alkaloids, chemical reactions were performed. The development of the color and / or characteristic precipitated was observed (Costa, 2001).

Thin layer chromatography

The thin layer chromatography was performed with different and specific mobile phase and reagents for each secondary metabolite (Table 1).

Bacterial samples

In this study, *Streptococcus mitis* ATCC 49456, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556 and *Enterococcus faecalis* ATCC 4082 were used, and the extracts and fractions were prepared in stock solution of 1600 µg / ml using solvent DMSO 20%.

Determination of antimicrobial activity - minimum inhibitory concentration (MIC) of the extract and fractions of *A. indica* A. Juss leaves

The Minimum Inhibitory Concentration (MIC) was determined using the dilution method on microplates according to standard M7-A6 Clinical and Laboratory Standards Institute (CLSI), with modifications.

Bacterial strains were maintained on Mueller-Hinton broth plus 50% glycerol and held at -20°C. After that, the bacterial strains were transplanted in 2 ml of Mueller-Hinton broth, incubated for 24 h at 37°C and were subsequently subcultured on solid medium (Blood Agar Base), incubated for 24 h at 37°C and maintained in refrigerator.

The bacterial suspensions were standardized by adding a culture prepared from the bacterial growth at 24 h elapsed MH broth at 37°C and subsequently, after stirring, a suspension aliquot was transferred to another tube containing sterile saline (PBS) until turbidity comparable to McFarland scale suspension corresponding to 0.5 tube unit (approximately 1.5×10^8 CFU/ml).

Next, the spectrophotometric reading was performed at 620 nm absorbance ranging from 0.10 to 0.15, which correspond to 1.5×10^8 CFU/ml. Subsequently, it was diluted on tube with sterile saline (PBS), to obtain an inoculum of bacteria *S. mutans, S. mitis, S. sanguinis* and *E. faecalis* the concentration of 5×10^5 CFU / ml used in the tests (Kawashima et al., 2003).

The plate's holes (96 wells) were filled with 80 µl of Mueller-Hinton broth. Then, 100 µl of solutions of plant extractives were added and serial dilutions carried out at 400 to 0.19 µg/ml for the concentration of 1600 µg/ml. Additionally**,** 20 µl of micro-organisms suspensions were distributed in each well of microplate. As a positive control was used chlorhexidine to 59 µg/ml for *S. mutans, S. sanguinis* and *S. mitis* and 14.75 µg/ ml for *E. faecalis*, and the negative control DMSO 20%.

Control of the culture medium, bacterial growth and plant extractives were also performed. The microplates were incubated at 37°C for 24 h. In each microplate two plant extractives were tested in duplicate. All tests were performed in triplicate.

RESULTS

Phytochemical screening

The results obtained on the phytochemical screening are presented in Table 2.

Thin layer chromatography

The results were positive for terpenes in the ethyl acetate extract; and the fractions resulting from the hydroethanol extract, which are hexane and ethyl acetate fraction; flavonoids in the ethyl acetate extract, hexane hydroethanolic extract and its fractions, n-butanol, ethyl

Table 3. Thin layer chromatography.

Extracts and fractions	Secundary metabolites
Hydroethanol extract	Flavonoids
Ethyl acetate extract	Flavonoids and terpenes
Hexane fraction	Flavonoids and terpenes
Ethyl acetate fraction	Flavonoids and terpenes
Water fraction	Flavonoids
n-Butanol fraction	Flavonoids

Table 4. Determination of the antibacterial activity and the minimal inhibitory concentration (MIC) in µg/ml for *S. mitis*, *S. sanguinis*, *S. mutans* and *E. faecalis* by dilution technique in microplate using resazurin as developer.

	Micro-organism	*S. mitis*	*S. sanguinis*	*S. mutans*	*E. faecalis*
MIC (µg/ml)	Hydroethanolic extract	400	≥400	≥400	50
	Hexane fraction	200	≥400	400	>400
	Ethyl acetate fraction	**100**	400	400	>400
	N-butanol Fraction	**50**	**100**	**50**	>400
	Aqueous fraction	400	≥400	≥400	**25**
	Extract ethyl acetate	**50**	**100**	**50**	>400
	Ampicillin	-	-	-	-
	Chlorhexidine	3.688	3.688	19.530	14.75

acetate and aqueous. Table 3 shows these results.

Antimicrobial activity

According to tests, the bacterial strains were sensitive to extracts and fractions of *A. indica* A. Juss leaves. The results with the MIC values are shown in Table 4.

DISCUSSION

More and more people in developed countries apply traditional medicine for most health care (Houghton, 1995). The use of medicinal plants for the treatment of various diseases including bacterial and fungal infections is common in Brazil (Carvalho et al., 2009; Pereira et al., 2007; Schubert et al., 2007). Worldwide, many search groups that use plants detect secondary metabolites with antimicrobial properties in an attempt to find new antimycobacterial or antifungals compounds (Cos et al., 2006; Soberón et al., 2007; Rangasamy et al., 2007).

There are several factors that can interfere qualitatively and quantitatively in the results, in the metabolites presence, in the antimicrobial activity, and one of them can be seasonal factor, which may be considered as the period of leaves collection. Therefore, the results can be related to low production of active metabolites against these microorganisms, thus, it is suggested further study and could start the study conducting the tests in all seasons.

The results of the phytochemical screening and thin layer chromatography confirms the presence of flavonoids and saponins, which justifies this plant's biological activities as anti-inflammatory, antimicrobial, antioxidant, antiviral, among others.

Observing the composition of the extracts and fractions, it can be said that the ethyl acetate extract, hydroethanol extract, the hexane fraction and ethyl acetate extract from hydroethanol extract showed terpenes and flavonoids in the composition. The n-butanol and aqueous fraction obtained positive result only for flavonoid, thus observed the difference in composition of the extracts and fractions tested. This result corroborates with the study of Mossini et al. (2005), which describes the presence of terpenes and phenolic compounds.

According to Gibbons (2004) and Van Vuuren (2008), crude extracts of natural products with an MIC lower than 1000 µg/ml are considered relevant and extracts with a MIC below 100 µg/ml are considered promising as potential antimicrobial agents. Therefore, based on these studies, it can be said that the n-butanol fraction and the ethyl acetate extract achieved a promising results with a MIC of 50 µg/ml to the strains *S. mitis* and *S. mutans*. These same samples showed MIC = 100 µg/ml for *S. sanguinis*, being also regarded as promising potential. For the *S. mitis*, the hexane fraction showed MIC = 200 µg/ml, which may be considered relevant, the hydroethanolic extract and aqueous fraction showed an

MIC = 400 µg/ml, also showing a considerable result.

Regarding the *S. sanguinis*, the ethyl acetate fraction showed MIC = 400 µg / ml, hydroethanolic extract, hexane fraction and aqueous fraction showed MIC ≥ 400 ug/ml, and can also be considered as relevant antimicrobial agents. For *S. mutans* the hexane fraction and ethyl acetate fraction showed a MIC = 400 µg/ml, hydroethanolic extract and aqueous fraction MIC ≥ 400 µg/ml, considered as an important result.

The *E. faecalis* results regarding the hydroethanolic extract and the aqueous fraction samples were promising, because it had, respectively, a value of MIC of 50 µg/ml, and MIC = 25 µg/ml, highlighting the activities of these vegetable samples for this strain. The remaining samples presented an MIC> 400 µg/ml, which is considered relevant.

Thus, the results corroborate with studies (Patel et al., 1988) which reports that the *A. indica* extract is a powerful inhibitor agent against the increase and establishment of micro-organisms that cause infectious diseases in the oral cavity, wherein *S. mutans* is the main causative agent of caries.

The activity of the aqueous fraction and hydroethanolic extract against *E. faecalis* are highlighted, with MIC of 25 µg/ml and MIC of 50 µg/ml, respectively, emphasizing these growth inhibition concentration values.

Conclusion

The positive antimicrobial activity of neem against the oral strains studied could be due to the presence of flavonoids and saponins, which justifies this species has biological activities as anti-inflammatory, antimicrobial, antioxidant, antiviral, among others. Considering the relevant MIC values of n-butanol fraction and ethyl acetate extract, especially for *S. mitis* and *S. mutans,* it was possible to observe that these samples have some active compounds that caused inhibition of their growth. These results encourage the continuation of the studies further isolation of this substance for a future development of herbal medicine.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Prospecting of efficient rhizobia for peanut inoculation in a Planosol under different vegetation covers

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Peanut (*Arachis hypogaea* L.) is capable of establishing an efficient symbiosis with a wide range of rhizobia, nevertheless, the inoculation of this legume is under used due to lack of information on the bacteria naturally established in soils and the agronomic and economic viability of the practice. The aim of this study was to evaluate the diversity and the symbiotic efficiency of peanut rhizobia from a Planosol under different vegetation covers (native vegetation, sugarcane cultivation and fallow after sugarcane cultivation), located in Zona da Mata, Northeast of Brazil. A total of 177 isolates were obtained from peanut nodules, most of them having the characteristic of rapid growth and acidification of the Yeast Mannitol Agar (YMA) medium. The isolates obtained from soil samples with native vegetation cover showed higher relative abundance, higher species richness and greater uniformity of morphological groups of rhizobia. Eighteen isolates were evaluated for their ability to nodulate peanuts, of which 13 have been authenticated and submitted to evaluation of symbiotic efficiency in pots with soil. The isolate 23M showed the best symbiotic performance in pots with soil. In the field, plants inoculated with the isolate 23M showed better performance than non-inoculated plants, in addition to a grain yield (kg ha^{-1}) similar to that of plants inoculated with recommended bacteria or receiving 200 kg ha^{-1} N.

Key words: Bacteria of legume nodules, biological nitrogen fixation, diversity, grain legume, sugarcane field reform.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a legume of major global importance, being grown in Asia, Africa, North America and South America, where it originated (Sujay et al., 2012; Wang et al., 2012; Mokgehle et al., 2014; Pozzi et

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al., 2014). In the peanut production system, one of the factors that most influence yield is an appropriate management of fertilization, especially nitrogen. To obtain high yields, this crop removes a high amount of the nutrient from the soil, and accumulates it in its biomass (Ambrosano et al., 2013). Hence, practices that enable the nitrogen supply are essential for a rational cultivation of peanuts. Inoculation with N_2-fixing bacteria (rhizobia) is an economical and environmentally advantageous option to favor the supply of N to plants. The symbiosis of the peanut with these bacteria can promote an increased grain yield, in addition to being an alternative for the partial or total replacement of nitrogen fertilizers (Torres Júnior et al., 2014). However, in Brazil, the peanut inoculation is not widely used, mainly due to lack of technical information aimed at the characterization of the agronomic and economic viability of the practice. The reason for this is that the peanut has the ability to nodulate with a wide variety of native rhizobia, which often reduces the responses to inoculation (Borges et al., 2007; Marcondes et al., 2010). In this sense, obtaining bacteria with high efficiency in the combination with peanut plants may represent a strategy to increase the interest of producers for the adoption of the inoculation technique (Torres Júnior et al., 2014).

In recent years, prospecting of effective rhizobia for the inoculation of various legumes has been carried out in Brazilian soils, yielding promising results for calopo (Calheiros et al., 2013; Calheiros et al., 2015), velvet bean (Lima et al., 2012) and cowpea (Chagas Junior et al., 2010), among others. Nonetheless, few studies have evaluated the efficiency of peanut isolates (Hoffman et al., 2007; Torres Júnior et al., 2014; Sizenando et al., 2016).

The obtention of rhizobia strains efficient in biological nitrogen fixation (BNF) comprises different test conditions, making the bases for recommendation of strains, which is divided into four stages: 1) selection under controlled conditions in the laboratory; 2) selection under sterile conditions in Leonard jars; 3) selection in pots with unsterilized soil; and 4) selection under field conditions (Howieson and Dilworth, 2016). The obtention of the collection of native isolates and the pre-selection of effective bacteria, generate genetic resources for implementation of programs for the recommendation of new strains (Rufini et al., 2013).

Given these considerations, this work aimed to select promising isolates for peanut inoculation in the region of Zona da Mata, Pernambuco state, Brazil. The soils of this region are home to a great diversity of bacteria capable of nodulating this legume, including the possibility of yet unknown species (Lyra et al., 2013). Thus, it was initially carried out, the prospecting of rhizobia in a Planosol with three different vegetation covers, in order to obtain a collection of isolates adapted to the edaphoclimatic conditions of the region and to assess the diversity of naturally established populations. With the obtained isolates, successive experiments were performed under sterile conditions, in pots with soil and under field conditions to evaluate the symbiotic capacity of the rhizobia obtained.

MATERIALS AND METHODS

Capture of naturally established rhizobia in soil with three different vegetation covers

To obtain the nodules and isolate the bacteria, an experiment was conducted in a greenhouse growing peanut (*Arachis hypogaea* L.) in pots with a capacity of 4.5 kg of soil. Samples were collected in 0 to 20 cm layer of a Haplic Planosol (Embrapa, 2013), with three different vegetation covers: 1) sugarcane cultivation; 2) fallow after sugarcane cultivation; and 3) native vegetation (Atlantic Forest). The samples were collected in the municipaility of Itambé (7°26' 49" S and 35°14' 27"W, 179 m), located in the region of Zona da Mata, Pernambuco state, Brazil. The climate, according to Köppen classification, is tropical rainy As', with average annual rainfall of 1400 mm and temperature of 24.2°C.

The collected soil samples were air-dried, sieved (5 mm) and distributed in the pots. Subsamples were taken to determine the chemical and physical characteristics of the soil under each vegetation cover (Table 1), following the methods recommended by Embrapa (2009). The peanut seeds (cultivar BR-1) were surface-sterilized (ethanol 70%, for 30 seconds, and sodium hypochlorite, for 1 min) and then washed in sterile distilled water. Each pot received four seeds, leaving one plant per pot after 10 days. A completely randomized block design was adopted; the treatments consisted of three types of vegetation covers, with 7 replications each. Plants were harvested at 50 days after emergence. The roots were separated from the shoots and the nodules were detached and packed in tubes with silica gel for drying and conservation.

For the rhizobia isolation, 6 nodules of each plant were randomly selected. These nodules were rehydrated in autoclaved distilled water for 40 min, being surface-disinfected (70% ethanol, for 1 min, and 5% sodium hypochlorite, for three minutes) and then washed 10 times with sterile distilled water. The disinfected nodules were pressed into a Petri dish containing yeast mannitol agar medium (YMA, pH 6.8) (Vincent, 1970) with 25 mg kg^{-1} (w/v) of Congo red (Somasegaran and Hoben, 1994). The plates were incubated at 28°C until the appearance of bacterial colonies. For purification of isolates, the colonies were inoculated successive times in YMA medium containing bromothymol blue indicator, until the obtention of pure culture.

The following characteristics of the isolates were observed in YMA medium with bromothymol blue: time required for the appearance of isolated colonies (rapid: within three days; intermediate: four to five days; slow: six days or more); change in the pH of the medium after cell growth (acid, neutral or alkaline); colony size (< 1 mm, 1 to 2 mm and > 2 mm); transparency (translucent or opaque); elevation (flat or convex); color (white or yellow); shape and edge of the colony; appearance of the mucus of colonies (homogeneous or heterogeneous) and type of mucus, which was sorted as butyric or viscous (Vicent, 1970).

The characterization data were encoded in a binary matrix. With this, the simple matching coefficient was calculated and the clustering of strains was performed through NtsysPC program, using the UPGMA algorithm and the Jaccard similarity matrix. A dendrogram was made with the purpose of separating the isolates in groups with higher similarities (Rohlf, 2000). The results of the cluster analysis were used to calculate diversity (Shannon H), richness (Margalef), and evenness (Pielou) indices for the soils and species, where each morphological group, at 75% similarity, was considered as one operational taxonomic unit. The PAST

Table 1. Characteristics of the Planosol under different vegetation cover in the States of Pernambuco (PE), Brazil.

Soil characteristics	Vegetation cover		
	Native vegetation	Sugarcane	Fallow
pH (H_2O)	6.1	6.2	6.2
Ca^{2+}(cmolc dm^{-3})	6.8	1.65	1.5
Al^{3+}(cmolc dm^{-3})	0	0	0
Mg^{2+}(cmolc dm^{-3})	6.2	2.6	2.5
Na^{+}(mg dm^{-3})	0.002	0.001	0.003
K^{+}(mg dm^{-3})	0.010	0.023	0.022
P (mg dm^{-3})	1.08	0.04	4.30
Dg (kg.dm^{-3})	1.22	1.18	1.24
Sand (%)	70.44	52.58	87.56
Clay (%)	17.58	34.40	7.24
Silt (%)	11.99	13.03	5.20

pH in water (1:2.5); P, K and Na extracted by Mehlich-1; Ca, Mg and Al extracted by 1 mol L^{-1}. All determinations according to Embrapa (2009).

(palaeontological statistics) program was used to perform cluster analysis and diversity indices calculation (Hammer et al., 2001).

The ability of nodulate the original host was confirmed for two representative isolates of each of the phenotypic groups obtained, by cultivating peanut in monoxenic conditions, using Leonard jars (Vincent, 1970) containing sterile substrate (sand and vermiculite in a 1:1 (v/v) ratio). It was adopted a randomized complete block design with 20 treatments (18 isolates, a control inoculated with the strain SEMIA 6144, *Bradyrhizobium* sp., and an absolute control without inoculation) and three replications.

Before sowing, peanut seeds were surface-sterilized, as described in the step of obtention of nodules. To prepare the inoculants, the rhizobia isolates and the control strain were cultured in YM medium for a suitable period of growth for each bacterium. At the time of sowing, 1 mL of culture broth with approximately 10^9 viable cells was applied to each seed. After the fall of the cotyledon leaves, a nitrogen-free nutrient solution was applied weekly (Norris and T'Mannetje, 1964). Plants were harvested at 45 days after emergence, being assessed the nodulation ability of the inoculated isolates. The isolates that were able to form viable nodules (pink or red color in the interior, showing the presence of leghemoglobin) were authenticated as peanut rhizobia (bacteria capable of nodulating and fixing N in symbiosis with legumes).

Symbiotic efficiency of isolates in pots with soil

Three independent and simultaneous experiments were conducted under greenhouse conditions to evaluate the symbiotic efficiency of the authenticated peanut isolates, in Leonard jars with sterile substrate, as in the experiment described above. In each experiment, the isolates were tested, by cultivating peanuts in pots containing soil samples, collected in areas with one of the three types of vegetation cover (sugarcane cultivation, fallow and native vegetation) of the Planosol as described above.

A completely randomized block design was adopted, in which the effects of both the non-inoculation of peanut and the inoculations with different strains of rhizobia (the isolates obtained and authenticated as described above and the recommended strains BR426 and SEMIA 6144, applied alone) on the nodulation and shoot biomass production of peanut (cultivar BR-1) were compared with the effects of fertilization with nitrogen fertilizer (dose equivalent to 100 kg ha^{-1}). Nitrogen fertilization was provided in the form of urea in a single dose. All treatments received phosphate and potassium fertilization in the form of triple superphosphate and potassium chloride, respectively, according to the fertilization recommendation for the peanut crop in the state of Pernambuco (Ipa, 2008). The same procedures were adopted for disinfection of seeds, preparation and application of inoculants and supply of nutrient solution as described above.

Plants were harvested at 45 days after emergence, being evaluated; the nodulation (number and biomass of nodules) and the shoot biomass production. Statistical analyses were performed using the computer program Sisvar (Ferreira, 2008). The variables were submitted to analysis of variance by F test , where F was significant, the means were compared by Tukey test at 5% probability.

Symbiotic efficiency in the field

An experiment was conducted under field conditions in the area of fallow after sugarcane cultivation, to test the isolate that provided the best dry biomass yield in the experiments under controlled conditions. This area was chosen because the cultivation of legumes that are able to perform BNF during soil fallowing at the sugarcane field reform is interesting to restore the natural soil fertility (Ambrosano et al., 2013).

In addition to this isolate, treatments with inoculations strains SEMIA 6144 and BR 426 were used, plus two controls without inoculation, being one with nitrogen (200 kg ha^{-1} of urea in planting). The experimental design was randomized blocks with six replications, in plots of 2 m x 2 m. The peanut cultivar BR-1 was sown in rows spaced 0.5 m apart, with 8 to 10 plants per linear meter. The experiment was harvested 90 days after germination and at harvest, the dry biomass of shoots and the grain yield were evaluated.

Statistical analyses were performed using the computer program Sisvar (Ferreira, 2008), the variables were submitted to analysis of variance by F test where the F was significant, the means were compared by Tukey test at 5% probability.

RESULTS AND DISCUSSION

From the nodules collected in the capture experiment, 177 bacterial isolates were obtained from the soil

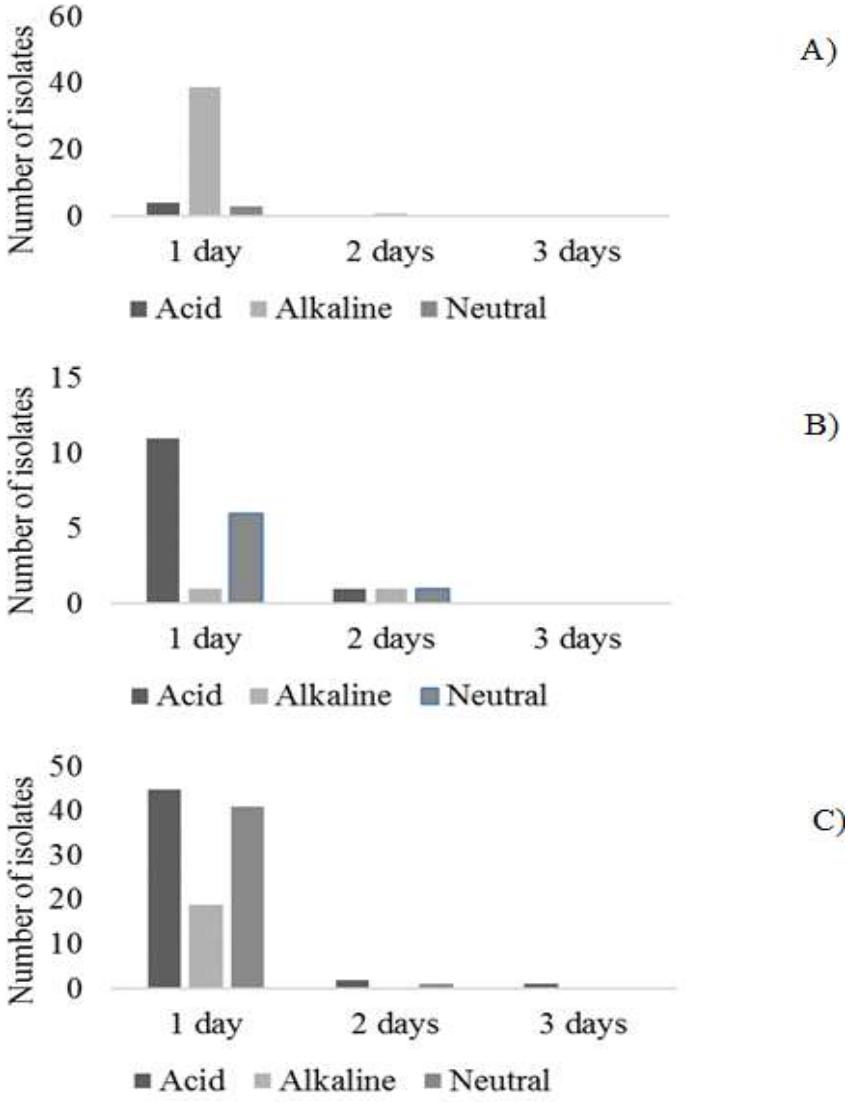

Figure 1. Number of bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.A= native vegetation, B = sugarcane and C= fallow.

samples of different vegetation covers, 47 of these from the area with native vegetation cover, 109 from the fallow area and 21 from the area with sugarcane cultivation.

Most studies report that peanut-nodulating rhizobia are belonging to the genus *Bradyrhizobium* (Steenkamp et al., 2008), which shows characteristics of slow growth and alkaline reaction in YMA culture medium (Wong et al., 1988). However, populations of isolates with rapid growth and acidification are apparently more common in soils from different regions of Brazil (Lyra et al., 2013; Torres Júnior et al., 2014). In contrast to these results, all isolates obtained in the Planosol showed rapid growth (up to three days in YMA culture medium with bromothymol blue incubated at 28°C), while majority (61%) presenting a metabolism which alkalizes the culture medium. This shows that the populations of peanut rhizobia naturally established in different soils, have variable characteristics according to the edaphoclimatic conditions to which they have adapted.

This type of original soil cover is conditioned to the differences in the pH reaction, of the culture medium of isolates. Among the isolates from the area covered with native vegetation, there was predominance of bacteria which promote alkaline reaction (Figure 1A). In the area with sugarcane cultivation, there was predominance of rhizobia of acid reaction (Figure 1B). Finally, in the fallow area, the proportion of acidifying, alkalizing and neutral bacteria were similar (Figure 1C). As for the size of the colonies, 75 isolates (42%) had colonies with a diameter less than 1 mm, 84 isolates (47%) showed colonies

Figure 2. Similarity dendrogram constructed from the cultural characteristics of rhizobia isolated from peanut grown in a Planosol under different vegetation covers in the States of Pernambuco (PE), Brazil.

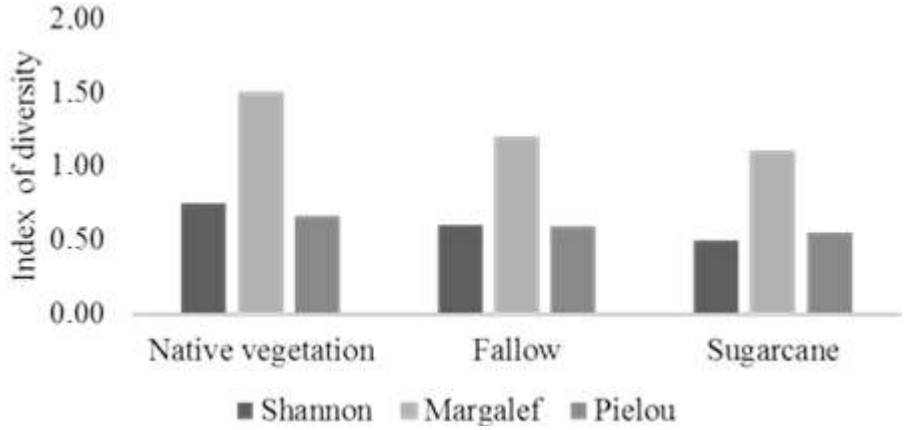

Figure 3. Shannon, Margalef and Pielou indices for bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.

between 1 and 2 mm, and 18 isolates (11%) had colonies with more than 2 mm diameter. All colonies showed white color, among which predominated the producers of medium and high amounts of exopolysaccharides.

The clustering of isolates from the characteristics thereof in the culture medium resulted in the formation of 9 groups with 75% similarity (Figure 2). This clustering based on phenotypic characterization is an important step in the process of selection of new isolates, as it enables a reduction in the number of bacteria, that will be evaluated in the process of authentication and symbiotic efficiency in sterile substrate. Furthermore, it makes possible to estimate the diversity of rhizobial populations in each studied area, from the indices calculation.

The type of soil cover conditioned differences, is in the diversity of bacterial populations of peanut nodules (Figure 3). According to the indices of Shannon (1948), Margalef (1958) and Pielou (1977), the populations naturally established in the area under native vegetation had greater diversity, richness and evenness of morphological groups in relation to the populations of the other two areas. The effects of changes in the land use

Table 2. Growth characteristics or authenticated bacterial nodule isolates from peanut grown in a Planosol under different vegetation covers.

Isolates	Vegetation cover	Growth time	pH reaction	Production of exopolysaccharides
6A1D	Fallow	Fast	Neutral	Medium
23M2A	Native vegetation	Fast	Neutral	Medium
16M1B	Native vegetation	Fast	Neutral	Medium
6M1E	Native vegetation	Fast	Alkaline	Medium
6A1C	Fallow	Fast	Neutral	Medium
16M1C	Native vegetation	Fast	Neutral	Medium
22M2B	Native vegetation	Fast	Neutral	Medium
23M2C	Native vegetation	Fast	Neutral	Medium
11C1A	Sugarcane	Fast	Acid	Medium
4A1F	Fallow	Fast	Acid	High
18C1C	Sugarcane	Fast	Acid	Medium
6A1A	Fallow	Fast	Neutral	Medium
23M	Native vegetation	Fast	Alkaline	Medium

system on populations of soil microorganisms are conditioned by the edaphoclimatic particularities of each environment. Contrary to the results found in this research, many studies point to the existence of populations with lower diversity in soils under native vegetation. For example, in the Amazon, the bacterial communities of grazing and agricultural areas showed greater diversity among different land use systems, even when considering different species of macrosymbionts (Jesus et al., 2009). In a study conducted in northeastern China, communities of soybean-nodulating bacteria had higher diversity in soils covered with grassland compared with those coming from fallowing soils, or making use of monoculture or crop rotation (Yan et al., 2014).

The lower diversity found in the area cultivated with sugarcane in the present study may be due to the large anthropic interference and the consequent process of degradation suffered by this ecosystem, with deforestation of the Atlantic Forest and cultivation of the sugarcane monoculture for several years. Despite the low diversity, the fact that some rhizobia groups persist in this ecosystem, as in others in a degraded state, is of fundamental importance, since the biological nitrogen fixation process mediated by these microorganisms can provide environmental resistance against an impact or stress, contributing to the resilience of degraded areas (Saturno et al., 2015).

Among the isolates tested in monoxenic conditions, 13 re-nodulated the peanut plants, confirming to be rhizobia. All of these isolates are fast growing and producing exopolysaccharides, with differences in relation to the pH change in YMA medium (Table 2) and high variability as regards its symbiotic efficiency in the peanut cultivated in pots with soil (Table 3). In general, the peanut plants showed better development, when grown in the soil collected in area under sugarcane cultivation, with an average 21% higher than the average biomass yield of the peanut cultivated in the soil of the Atlantic Forest and 25% higher than the average yield in the fallowing soil. The highest peanut yield in the soil with sugarcane production could be attributed to the considerable chemical fertilization normally used in the system, however there were no differences in the availability of major nutrients in the soil of this area in relation to others (Table 1). Fertilezers and lime represent an important secondary source of micronutrients for the soil and can be an important in the soil fertility (Carvalho et al., 2012). Possibly, the availability of some efficient nutrient absent in our parameters analysed, such as Mn, Zn, Cu and Fe, conditioned the highest yield of peanut grown in soil with sugarcane production.

The symbiotic performance of strains, assessed by the shoot biomass production of inoculated plants, varied according to the soil in which the peanut was grown. Bacteria from the soil covered with native plants were more efficient when inoculated in peanuts cultivated in the soil, collected in the same location (isolates 23M and 23M2A), and also grown in the soil collected in the area with sugarcane cultivation (16M1B). However, when the peanut was grown in soil cultivated with sugarcane, the best performance was obtained in plants inoculated with the isolate 11C1A, native of sugarcane area. Considering the overall average yield of each isolate in the three land use systems, it appears that the isolate 23M had the highest average shoot biomass yield (2.118 g plant^{-1}), which is approximately 10% higher than the control and received nitrogen fertilization which is a promising isolate for the production of inoculants.

Santos et al. (2005) evaluated the efficiency of peanut-nodulating rhizobia, isolated from soils in northeastern Brazil, and obtained a dry matter accumulation ranging from 1.54 to 2.59 g plant^{-1}, values which is close to the results of this study.Torres Júnior et al. (2014) evaluated the shoot biomass yield of peanut cultivars, inoculated

Table 3. Dry biomass productivity of peanut inoculated with diffetent isolates in a Planosol under different vegetation covers.

Inoculation	Covers vegetation			
	Native Vegetation	Sugarcane	Fallow	Overall average
	----------Dry biomass productivity t ha^{-1}----------			
Control	1.908^{ab}	2.108^{ab}	1.772^{ab}	1.929
Control with N	1.678^{b}	2.422^{ab}	1.65^{b}	1.917
6A1D	1.8a^{b}	2.182^{ab}	1.978^{ab}	1.987
23M2A	1.93^{ab}	2.567^{ab}	1.71^{ab}	2.069
16M1B	1.783^{ab}	2.254^{ab}	2.008^{a}	2.015
6M1E	1.663^{b}	1.99^{b}	1.777^{ab}	1.810
6A1C	1.848^{ab}	2.44^{ab}	1.745^{ab}	2.011
16M1C	1.888^{ab}	2.192^{ab}	1.91^{ab}	1.997
22M2B	2.024^{a}	2.534^{ab}	1.575^{b}	2.044
23M2C	1.865^{ab}	2.408^{ab}	1.725^{ab}	1.999
11C1A	1.827^{ab}	2.785^{a}	1.712^{ab}	2.108
4A1F	1.82^{ab}	2.232^{ab}	1.72^{ab}	1.924
18C1C	2.067^{a}	2.567^{ab}	1.622^{b}	2.085
6A1A	2.068^{a}	2.298^{ab}	1.555^{b}	1.974
23M	2.353^{a}	2.103^{ab}	1.898^{ab}	2.118
BR 426	1.468^{c}	2.098^{ab}	1.815^{ab}	1.794
SEMIA 6144	1.54^{b}	1.973^{b}	1.928^{ab}	1.928
Average	1.855	2.338	1.771	1.988

Means followed by the same letter do not differ significantly by the Tukey test at 5% probability.

with rhizobia isolates, which originated in the southeast of Brazil and, similar to our results found in a wide variation, in the efficiency of isolates. This has also been observed for rhizobia isolates, inoculated in other legumes (Lima et al., 2012; Calheiros et al., 2013). Such variability in symbiotic system which has the ability of collections of rhizobia, is a result of high biodiversity of these microorganisms, associated with biotic and abiotic factors. Studies have shown that the symbiotic association is affected by several factors such as soil acidity, aluminum toxicity (Campanharo et al., 2010), salinity (Medeiros et al., 2008), low soil fertility (Sulieman and Tran, 2015), phosphorus and molybdenum deficiency (Matoso and Kusdra, 2014), high soil temperatures (Ferrari et al., 1967) and low water availability in the soil (Andres et al., 2012). The use of microsymbionts adapted to different environmental conditions, combined with the correction of factors related to soil fertility, which may potentiate the effects of inoculation with diazotrophic bacteria.

Despite these promising results of some of the isolates obtained until the phase of screening tests in pots with unsterilized soil in the field test, only the strain BR 426 and the nitrogen treatment (200 kg ha^{-1}) showed shoot biomass production which is statistically higher than the control without inoculation and nitrogen fertilization. The plants inoculated with the test isolate (23M) did not differ statistically neither, from the treatment that received inoculation with the strain SEMIA 6144, nor from non-inoculated plants. According to Souza et al. (2008), the biomass accumulation is a strong indicator of the nutritional status of the plants and has been one of the variables used in studies for selecting rhizobia isolates with potential for BNF (Marcondes et al., 2010; Torres Júnior et al., 2014; Valetti et al., 2016).

However, the grain yield results revealed that, in the conditions under which the experiment was conducted, all inoculated treatments (with the recommended strains SEMIA 6144 and BR 426 and also with the isolate originated from the forest, 23M) were statistically superior than the control without inoculation and nitrogen fertilization. Moreover, the inoculation promoted the same grain production than the fertilization with 200 kg ha^{-1} urea. These results reveal that the test isolate (23M), when used as inoculant in peanuts, was able to increase grain yield by more than 780 kg ha^{-1} when compared to the treatment without inoculation and nitrogen fertilization (Table 4). Interestingly, this isolate features fastgrowth the alkaline metabolism in YMA medium; unusual features in bacteria of legume nodules which is different from the previously reported for peanut rhizobia in tropical soils (Lyra et al., 2013; Torres Júnior et al., 2014).

The practice of nitrogen fertilization has been increasingly used in production systems. The replacement

Table 4. Above groundbiomass and grain yield of peanut cv. BR 1, cultivated in a Planosol under fallow.

Treatment	Dry biomass productivity of aerial part (kg ha^{-1})	Productivity of grains (kg ha^{-1})
Control	509^{b}	1387^{b}
Urea 200 kg ha^{-1}	569^{a}	1802^{a}
Semia 6144	525^{ab}	1774^{a}
BR 426	588^{a}	1766^{a}
23 M	480^{b}	2180^{a}

Means followed by the same letter do not differ significantly by the Tukey test at 5% probability.

of chemical fertilizers by the use of efficient inoculants, contributes to the reduction of environmental impacts and production costs. Therefore, the lack of statistical difference between nitrogen control and test isolate (23M) suggests that this isolate can be used as an alternative to the use of nitrogen fertilizers in the northeast of Brazil.

Conclusions

Bacterial isolates of peanut nodules coming from soils of the Brazilian Zona da Mata have high morpho-physiological diversity. These isolate 23M showed a high potential for evaluation in network, testing for the recommendation of new strains for peanut inoculation.

Conflict of interest

The authors have not declared any conflict of interest.

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

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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 Sa442 R	5'-AATCTTTGTCGGTACACGATATTCTTCACG-3' 5'- CGTAATGAGATTTCAGTAGATAATACAACA -3'	108	Martineau et al. (1998)
icaA F icaA R	5'-CCTAACTAACGAAAGGTAG-3' 5'-AAGATATAGCGATAAGTGC-3'	1315	Vasudevan et al. (2003)
icaD F icaD R	5'-AAACGTAAGAGAGGTGG-3' 5'- GGCAATATGATGAAGATAC-3'	381	Vasudevan et al. (2003)
clfA F clfA R	5'-ATTGGCGTGGCTTCAGTGCT-3' 5'-CGTTTCTTCCCACGTAGTTGCATTTG-3'	292	Yeon-Soo-Seo et al. (2008)
clfB F clfB R	5'-ACATCAGTAATAGTAGGGGGCAAC-3' 5'-TTCGCACTGTTTGTGTTTGCAC-3'	205	Eidhin et al. (1998)
hla F hla R	5'-AGAAAATGGCATGCACAAAAA-3' 5'-TGTTGCGAAGTCTGGTGAAAA-3'	600	Wolz et al. (2000)
sarA F sarA R	5'- TTGCGCTAAATCGTTTCATTATTAA-3' 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

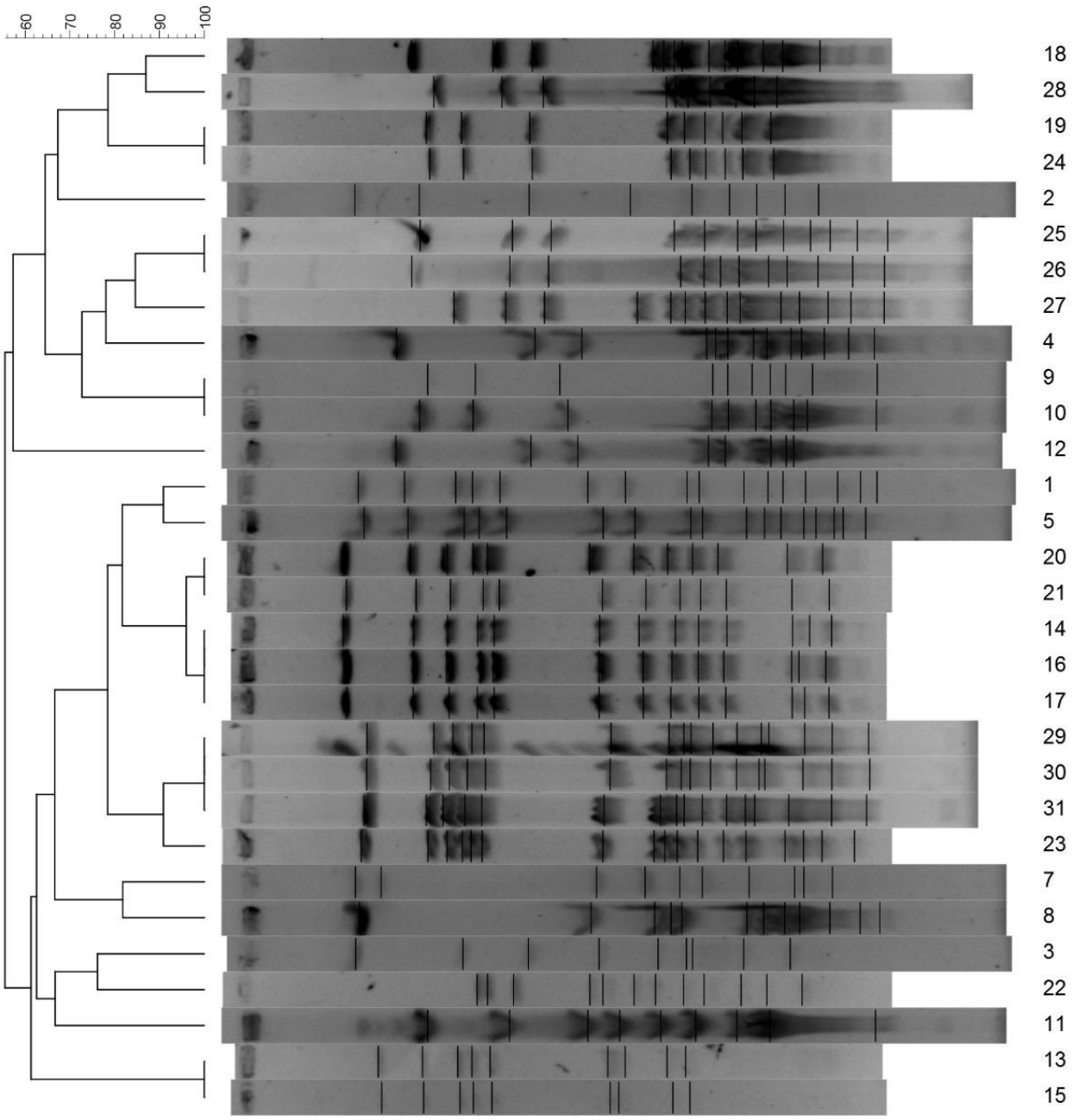

Figure 1. Pulsed-field gel electrophoresis (PFGE) of 32 *S. aureus* samples obtained from buffalo milk, milkers' hands and milking machine, digested with SmaI. Clustering were obtained by PFGE analysis in Bionumerics 7.1 software.

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.

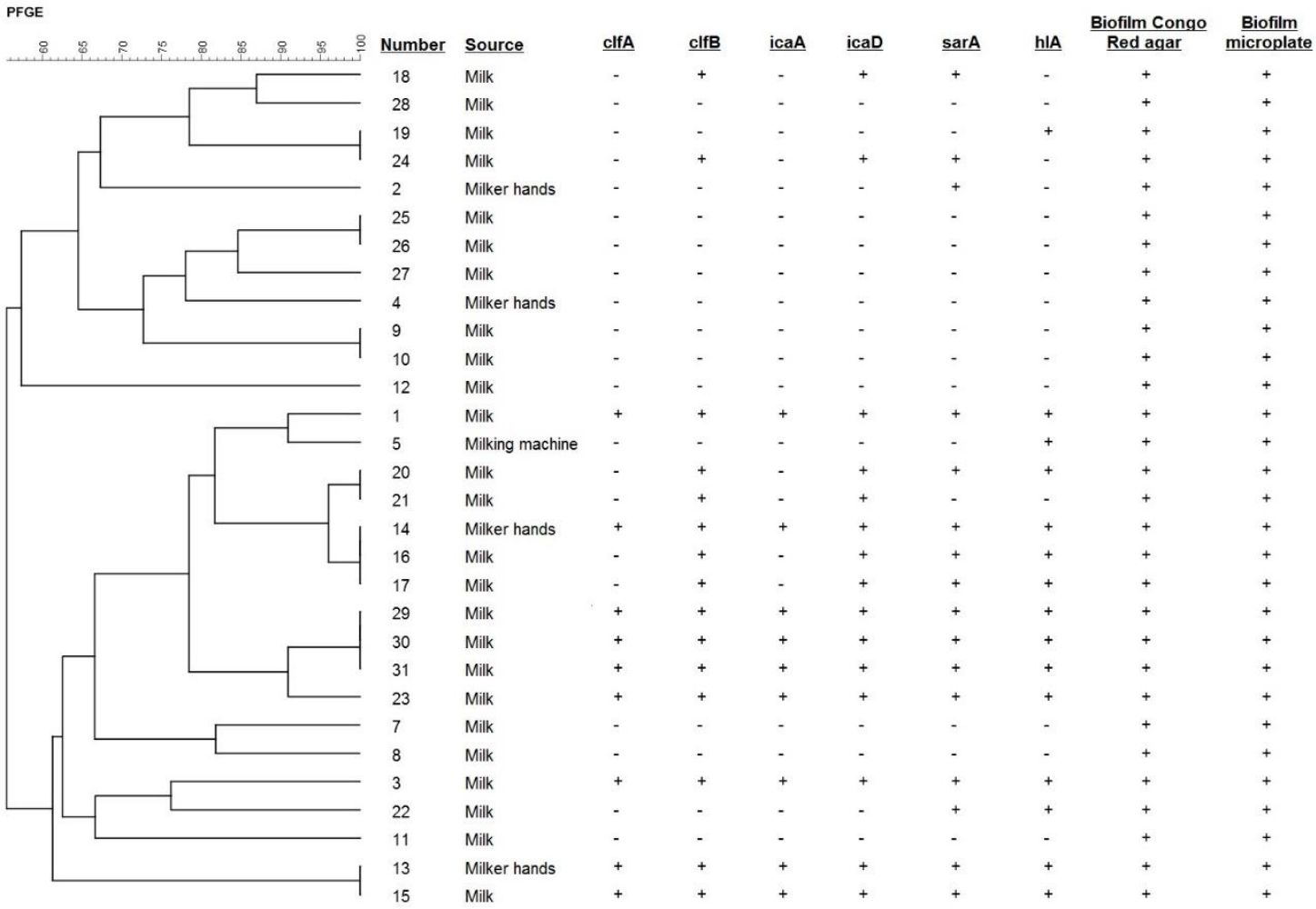

Figure 2. Dendrogram based on *S. aureus* pulsotypes detected by PFGE, followed by source of isolation, presence of virulence genes and biofilm formation capabilities on Congo Red Agar and microplate test created by Bionumerics 7.1 software.

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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