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Performance evaluation of oxacillin-resistant *Staphylococcus aureus* genotypes and *taxa* on human and animal blood agar culture media

Marcelo Fabiano Gomes Boriollo[1,2,3]*, Manoel Francisco Rodrigues Netto[1,3], Jeferson Júnior da Silva[3], Carlos Tadeu dos Santos Dias[4] and José Francisco Höfling[3]

[1]Laboratório de Farmacogenética e Biologia Molecular, Faculdade de Ciências Médicas, Universidade José do Rosário Vellano (UNIFENAS), Alfenas, MG, Brazil.
[2]Centro de Pesquisa e Pós-graduação em Ciência Animal, Área de Patologia e Farmacologia Animal, Universidade José do Rosário Vellano (UNIFENAS), Alfenas, MG, Brazil.
[3]Laboratório de Microbiologia e Imunologia, Departamento de Diagnóstico Oral, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (FOP/UNICAMP), Piracicaba, SP, Brazil.
[4]Departamento de Ciências Exatas, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo (ESALQ/USP), Piracicaba, SP, Brazil.

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The performance characteristics of growth, morphological aspects and hemolytic activities of oxacillin-resistant *S. aureus* (ORSA) strains were studied on twelve types of blood agar (BA) culture media (sheep, bovine, horse, rabbit and human). ORSA isolates were also previously characterized by isoenzymes genotyping and genetic and grouping analysis. Variations in the diameter of the colonies were detected among seven sets of BA media. In terms of morphology, 99, 53 and 98% presented shiny, yellow, and glossy colonies, respectively, regardless of the type of BAs. The rabbit BA favored the expression of hemolysins for most isolates (74%), followed by the human BA and other animal BAs. Certain BA media promoted the expression of hemolysins; however, the expression was correlated with a deficit in the colonial growth potential and *vice-versa*. The data point to the existence of two or more isolates genetically identical or highly related: (i) that either share or do not share the same wild species-specific phenotypes related to appearance without any influence from the external environment, and that are (ii) potentially virulent depending on the external environment. This study also suggestes the use of rabbit BA for the phenotypical characterization of *S. aureus*.

Key words: Colonial morphology, hemolysis, human and animal blood agar, oxacillin-resistant *Staphylococcus aureus*, clinical microbiology.

INTRODUCTION

The technical procedures of isolation and microbiological culture remain as the "*gold standard*" for clinical diagnosis of numerous bacterial infections, including the species *Staphylococcus aureus*. However, the characterization of certain microorganisms requires a blood source as a supplement in culture media. These culture media have been used routinely for the isolation and preliminary identification of *S. aureus* and other microorganisms of

medical importance (for example streptococci and enterococci) or even in subcultures preceding phenotypic tests for identification and antimicrobial susceptibility (Anand et al., 2000). In addition, culture media containing the defibrinated sheep, horse, pig or goat blood agar (BA) have been recommended for the isolation of *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Anand et al., 2000; Centers for Disease Control and Prevention, 1998; Gratten et al., 1994; Johnson et al., 1996; Sharp and Searcy, 2006). Furthermore, the phenotypic characterization of certain virulence factors in *S. aureus* (Kuroda et al., 2001) can be determined through use of blood agar culture media, for examining the determination of exotoxins (α, β, δ and γ-hemolysins) (Bohach et al., 1997; Bohach and Foster, 2000; Peacok et al., 2002; Sakoulas et al., 2002), which also have clinical significance in the development of human diseases (Yarwood and Schlievert, 2003).

Given the unfavorable recommendations for the microbial isolation or susceptibility testing, the potential safety risks to laboratory technical experts (e.g., risk of blood infections: Hepatitis B and HIV) and the low rate of bacterial isolation, human blood is not recommended for growing cultures in microbiological laboratories (e.g., human blood may contain anti-microbial agents and antibodies and it may inhibit the microbial growth or cause false haemolysis) (Anand et al., 2000; Centers for Disease Control and Prevention, 1998; CLSI document M07-A9, 2012; CLSI document M02-A11, 2012; Gratten et al., 1994; Johnson et al., 1996; Satzke et al., 2010). Although there is little data on the subject, in many developing countries, the preparation of bacterial culture media from expired human blood, from donors of blood transfusions, has been a common practice and is considered convenient and inexpensive. This practice has also been routinely employed in bacteriology laboratories from seven countries in the Asia-Pacific region, as mentioned in previous studies (Russell et al., 2006).

The blood considered for this purpose should be defibrinated while harvested or collected in bags containing anticoagulant, thus preventing the formation of clots. *Citrate phosphate dextrose* (CPD) is the commonly employed anticoagulant. In turn, citric acid has also been used in the food industry as an inhibitor of bacterial growth (Young and Foegeding, 1993; Phillips, 1999) and, for this reason, it has been considered inappropriate for use in culture media. In developed countries, commercial animal laboratories use magnetic stirrers to defibrinate the blood during collection procedures. However, this type of specialized equipment tends to be difficult to obtain in developing countries cases, the procedure for blood collection requires a sterile glass container containing glass beads *in situ*, which is gently agitated manually and rotated during the process of collection. This allows the binding of fibrin around the spheres to prevent clot formation. However, this practice displays limitations for small volumes of blood. According to Russell and associates, in Fiji, human BA is used routinely in bacteriological diagnostic laboratories, given the impracticality of establishing a reliable source of blood for research laboratories, despite the possibility of collecting animal blood in commercially available human blood donor bags containing CPD (Russell et al., 2006).

Taking into consideration the data from the literature about the need for isolation and microbiological and molecular characterization of microorganisms of medical interest, the purpose of the present research was to compare agar culture media supplemented with many types of citrated non-commercial human and animal blood sources and commercially available defibrinated sheep blood. We evaluated each BA in terms of their performance characteristics of bacterial growth and production of hemolysis *in vitro*, in a special manner, for a group of odontological patients and clinical environment (air) isolates of oxacillin-resistant *S. aureus* (ORSA). These isolates were previously characterized by isoenzymes genotyping (Multilocus Enzyme Electrophoresis - MLEE) and genetic and grouping analysis (that is, identification and genetic relationship among strains, *clusters* and *taxa*, usually established in molecular epidemiological tracking studies) to establish a possible correlation between phenotype and genotype.

MATERIAL AND METHODS

Microbiological sampling

A total of ninety-nine bacterial samples of ORSA, from the bacteria collection of the *Laboratório de Farmacogenética e Biologia Molecular*, *Faculdade de Ciências Médicas and Centro de Pesquisa e Pós-graduação* (UNIFENAS), Alfenas, MG, Brazil, were kindly provided and used for the present research. These samples were previously isolated from odontological patients and clinical environment (air) (*Faculdade de Odontologia*, UNIFENAS) and characterized using microbiological methods of identification [that is, stain of Gram, growth in chromogenic medium CHROMagar *Staphylococcus aureus*®, catalase test, coagulase test (Coagu-Plasma, Laborclin Produtos para Laboratórios Ltda.), clumping factor A test (Staphy Test, Probac do Brasil Produtos Bacteriológicos Ltda., Marnes La Coquette, France), fermentation of mannitol test and DNAse test (Winn et al., 2008)] and antimicrobial susceptibility testing [that is, diffusion disk (CLSI document M02-A11, 2012; CLSI document M100-S22, 2012) and confirmatory triage for resistance to oxacillin (CLSI document M07-A9, 2012)]. Genotyping of oxacillin- and resistant *S. aureus* was

*Corresponding author. E-mail: marcelofgb@yahoo.com.br

previously done by isoenzyme markers and genetic and grouping analysis.

Blood and culture media

Blood from clinically healthy animals (sheep, bovines, horses and rabbits) from the *Faculdade de Medicina Veterinária* and/or biotherium (UNIFENAS), in the absence of antibiotic therapy at the time of blood collection, was harvested directly (sheep, bovines and horses) or indirectly (rabbits, cardiac harvest) in sterile blood bags (CPDA1; citrate phosphate dextrose adenine, 3.27 g of citric acid monohydrate, 26.3 g of sodium citrate dihydrate, 2.51 g of monosodium phosphate dihydrate, 31.9 g of dextrose monohydrate, 0.275 g of adenine, injectable water qsp 1000 mL, sodium concentration 275 mM, Na/1000 mL, pH 5.6 ±0.3; MacoPharma, Mouvaux, France) by veterinary experts using aseptic techniques. Immediately after collection, the blood bags were transported (2-8°C) to the laboratory, centrifuged at 1,820 × *g* (2,500 rpm) at ambient temperature (Centrifuge Sorvall® RC3C Plus), producing a concentrated volume of red blood cells between 80 and 100 mL and then stored at 4±2°C until the time of use (< 30 days). Expired human blood samples (concentrated volume of red blood cells O^+, O^-, A^+, A^-, B^+, B^-, AB^+ and AB^- between 250 and 330 mL), collected from various donors in sterile manner (sterile CPDA1 blood bags; MacoPharma, Mouvaux, France) and stored at 4±2°C, were kindly provided by the blood bank from the *Hospital Universitário Alzira Velano* (HUAV), Alfenas, MG, Brazil. According to the clinical laboratory and serological information (Blood Bank HUAV), all the human blood samples tested negative for syphilis, AIDS, Chagas disease, hepatitis B, hepatitis C, HTLV-1, HTLV-2 and hemoglobin S. Prior to the microbiological tests and to ensure the sterility of the blood culture, 10 mL aliquots of each human and animal blood sample were transferred aseptically to BacTalert type bottles, incubated for seven days and analyzed using a BacT-ALERT® 3D system (bioMérieux Inc., Durham, NC).

Petri dishes containing blood agar (BA) culture media were prepared using standard methods (Oxoid Australia Pty. Ltd) using human and animal blood (5% vol/vol) and Columbia Agar Base (Oxoid Ltd.). A total of twelve types of BA [citrated sheep BA (CSBA), citrated bovine BA (CBBA), citrated horse BA (CHBA), citrated rabbit BA (CRBA), citrated human BA O^- (CHuBA O^-), citrated human BA O^+ (CHuBA O^+), citrated human BA A^- (CHuBA A^-), citrated human BA A^+ (CHuBA A^+), citrated human BA B^- (CHuBA B^-), citrated human BA B^+ (CHuBA B^+), citrated human BA AB^- (CHuBA AB^-) and citrated human BA AB^+ (CHuBA AB^+)] were produced and then stored at 4±2°C until used.

Characterization of ORSA on BA

For each isolate of oxacillin-resistant *S. aureus*, an inoculum was prepared from a direct suspension of bacterial colonies (approximately 1 to 2 × 10^8 CFU.mL^{-1} of 150 mM NaCl according to 0.5 on the McFarland scale) newly grown in BHI Agar (Brain Heart Infusion Agar, Difco™) at 35°C for 18 to 24 h. Using a pipette (Eppendorf Reference®, cat. # 4910 000.018. Eppendorf of Brazil Ltda. São Paulo, SP), aliquots of 5 μL of each bacterial inoculum were applied to the Columbia blood agar (5 isolates per culture medium) previously prepared in 90 × 15 mm Petri dishes (20 mL of culture media/dishes; medium height in each dish equal to 4±0.5 mm). These dishes were kept at ambient temperature up to 15 min to allow the complete moisture absorption and incubated in reversed mode at 35°C for 24 h. Soon after the incubation, the dishes were observed, and the results were recorded in terms of colonial morphology (by description and photos), colony diameter (mm) and production of hemolysis (by description and photos).

Hemolytic activity (*Pz*) was quantitatively and qualitatively characterized using a previously described methodology used for the characterization of virulence factors *in vitro* of *C. albicans* [that is, exoenzymatic activity (*Pz*) of secreted aspartyl proteinase and phospholipase on culture media, which seem to play an important role in pathogenicity of *C. albicans* and others *Candida* species] (Barros et al., 2008; Boriollo et al., 2009; Price et al., 1982): $Pz = dc/(dc + zp)$, where *dc* and *zp* correspond to the diameter (mm) of the colony and external diameter (mm) of the precipitation zone (hemolysis), respectively. These results were interpreted as follows: (*i*) $Pz = 1$, absence of hemolysis (index 0); (*ii*) $1 > Pz \geq 0.64$, positive hemolysis (index 1); and (*iii*) $Pz < 0.64$, strongly positive hemolysis (index 2). To ensure the typability and reproducibility of tests, *S. aureus* ATCC® 25923™ reference strain and commercially available defibrinated sheep BA (DSBA commercial. EBE Farma-Biológica Agropecuária Ltd. Niterói, RJ, Brazil) (the "positive control" for the morphological characteristics and virulence) and Columbia Agar Base (CAB) ("negative control" for the morphological characteristics and virulence) were included in the microbiological characterization tests (triplicate inoculum tests).

Statistical analysis

The results were also subjected to analysis of *one-way* variance (ANOVA) in a completely randomized factorial scheme design (culture media BA, taxonomic ranking and colonial phenotypes), and the averages were compared with Tukey's test ($\alpha = 0.05$) using SAS® version 9.2.

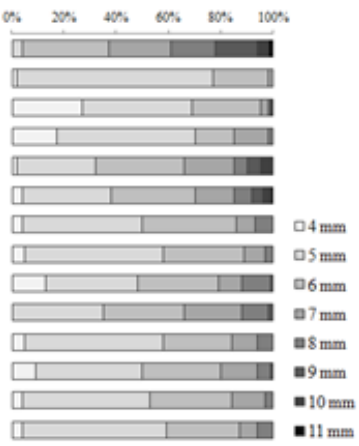
RESULTS

A population of 99 isolates of oxacillin-resistant *S. aureus*, previously characterized in 79 strains grouped in three *taxa* and 15 *clusters*, was evaluated for the size (mm of ∅) and the appearance of colonies (shiny or opaque, yellow or white, glossy or dry) and hemolysis activity (*Pz* obvious or faint) on thirteen different dishes of BA culture media (CSBA, CBBA, CHBA, CRBA, CHuBA O^-, CHuBA O^+, CHuBA A^-, CHuBA A^+, CHuBA B^-, CHuBA B^+, CHuBA AB^- and CHuBA AB^+) and a dish of CAB medium, in triplicate. In general, phenotypic variability could be observed between the different strains and even between different isolates belonging to the same strain. For example, there was variability in colony size and β-hemolysis activity among the isolates G20.44 and G18.100 that correspond to the same strain ET41, or still, there was variability in colony appearance, colony size and β-hemolysis activity among the isolates G18.104 and G20.45 that correspond to the same strain ET27, depending on the BA media. Such phenotypic variability was also observed among the strains ET41 and ET27 depending on the BA media (Supplemental Table 1).

Variations in the diameter of bacterial colonies (4-11 mm) grown on the different BA media tested could be observed in this population of isolates, including the reference strain of *S. aureus* ATCC® 25923™ (Table 1). Most bacterial isolates displayed a range of five (DSBA commercial, CSBA, CRBA, CBBA, CHuBA A^+, CHuBA A^-, CHuBA B^+, CHuBA B^-, CHuBA AB^+, CHuBA AB^-, CHuBA O^+ and CHuBA O^-) or six (CAB and CHBA) millimeters in

Table 1. Profiles of the diameters of colonies of oxacillin-resistant *S. aureus* [99 isolates (79 strains/ETs) and reference strain ATCC® 25923™] on 13 different types of blood agar plates and one Columbia agar base plate.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[F] CAB	-	4	33	24	17	16	4	2	5-11
[B, C] DSBA commercial	2	75	21	2	-	-	-	-	4-7
[A] CSBA	27	42	26	3	1	-	1	-	4-10
[A, B] CRBA	17	53	15	13	2	-	-	-	4-8
[E] CHBA	2	30	34	19	5	5	5	-	4-10
[E] CBBA	4	34	32	15	7	4	3	1	4-11
[D] CHuBA A^+	4	46	36	7	7	-	-	-	4-8
[D, C] CHuBA A^-	5	53	31	8	3	-	-	-	4-8
[D] CHuBA B^+	13	35	31	9	11	1	-	-	4-9
[E] CHuBA B^-	-	35	31	22	10	2	-	-	5-9
[D] CHuBA AB^+	5	53	26	10	6	-	-	-	4-8
[D] CHuBA AB^-	9	41	30	14	5	1	-	-	4-9
[D] CHuBA O^+	4	49	31	13	3	-	-	-	4-8
[D] CHuBA O^-	4	55	28	7	6	-	-	-	4-8



The letters [A, B, C, D, E] and [F] correspond to the Tukey grouping. The graphic to the right corresponds to the data from Table 1.

diameter, on average. However, significant differences ($p < 0.05$) were observed between BA media in seven situations:

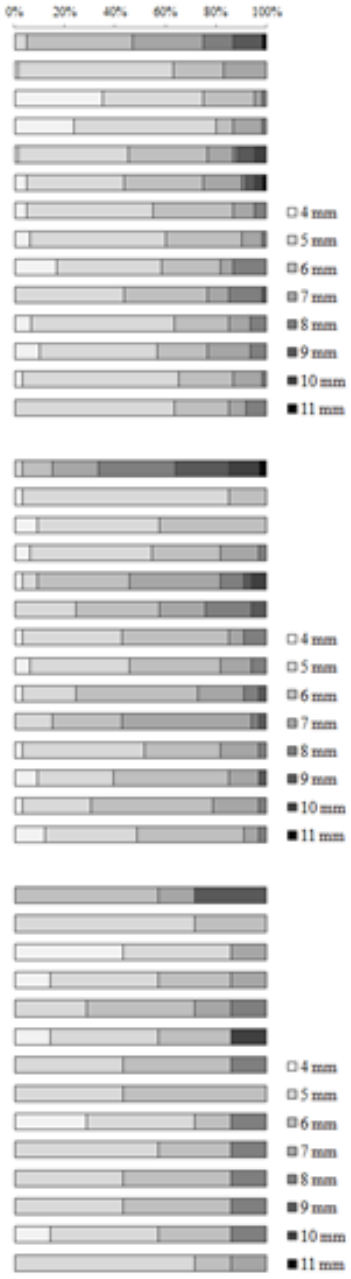
1. CSBA produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CBBA, CHBA, DSBA commercial, CHuBA O^-, CHuBA O^+, CHuBA A^-, CHuBA A^+, CHuBA B^-, CHuBA B^+, CHuBA AB^-, CHuBA AB^+ and CAB;
2. CRBA produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CBBA, CHBA, CHuBA A^-, CHuBA A^+, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^-, CHuBA O^+ and CAB;
3. DSBA commercial produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CBBA, CHBA, CSBA, CHuBA A^+, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^-, CHuBA O^+ and CAB;
4. CHuBA A^- produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CBBA, CHBA, CSBA, CRBA, CHuBA B^- and CAB;
5. CHuBA A^+, CHuBA AB^-, CHuBA AB^+, CHuBA B^+, CHuBA O^- and CHuBA O^+ produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CBBA, CHBA, CSBA, DSBA commercial, CRBA, CHuBA B^- and CAB;
6. CBBA, CHBA and CHuBA B^- produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in CSBA, DSBA commercial, CRBA, CHuBA A^-, CHuBA A^+, CHuBA AB^-, CHuBA AB^+, CHuBA B^+, CHuBA O^-, CHuBA O^+ and CAB; and
7. CAB produced variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in others BA media.

These variations in the diameter of bacterial colonies were also evaluated among the largest taxonomic ranks of ORSA [that is, *taxa* A (60 isolates/43 strains), B (33 isolates/30 strains) and C (7 isolates/6 strains) (Table 2) and among the smaller taxonomic ranks of ORSA (that is, *clusters* from I to XV)] (Table 3). The *taxon* A comprised isolates/strains with variations in the diameter of bacterial colonies different significantly ($p < 0.05$) those observed in *taxon* B. In turn, the *taxon* B comprised isolates/strains with variations in the diameter of bacterial colonies different significantly ($p < 0.05$) those observed in *taxon* C. The *taxa* A and C were considered statistically identical. As for the lower ranks, significant differences ($p < 0.05$) were observed between *clusters* in 10 situations:

1. *Cluster* XIV comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, V, VI, VII, VIII, IX, X, XI, XII, XIII and XV;
2. *Cluster* IV comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, V, VI, VII, IX, X, XI, XII, XIII and XV;
3. *Cluster* VIII comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, V, IX, X, XI, XII, XIII and XIV;
4. *Clusters* VI and XV comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, IV, V, IX, X, XI, XII, XIII and XIV;

Table 2. Profiles of the diameters of colonies within and among distantly genetically related populations (*taxa* A, B and C) of oxacillin-resistant *S. aureus* on 13 different types of blood agar plates.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[A] *Taxon A* (60 isolates / 43 strains [ETs])									
CAB	-	3	25	17	7	7	-	1	5-11
DSBA commercial	1	43	14	12	-	-	-	-	4-7
CSBA	21	24	12	2	1	-	-	-	4-8
CRBA	14	34	4	7	1	-	-	-	4-8
CHBA	1	26	19	6	1	4	3	-	4-10
CBBA	3	23	19	9	1	2	2	1	4-11
CHuBA A^+	3	30	19	5	3	-	-	-	4-8
CHuBA A^-	3	27	15	4	1	-	-	-	4-8
CHuBA B^+	10	25	14	3	8	-	-	-	4-8
CHuBA B^-	-	26	20	5	8	1	-	-	5-9
CHuBA AB^+	4	34	13	5	4	-	-	-	4-8
CHuBA AB^-	6	28	12	10	4	-	-	-	4-8
CHuBA O^+	2	37	13	7	1	-	-	-	4-8
CHuBA O^-	-	38	13	4	5	-	-	-	5-8
[B] *Taxon B* (33 isolates / 30 strains [ETs])									
CAB	-	1	4	6	10	7	4	1	5-11
DSBA commercial	1	27	5	-	-	-	-	-	4-6
CSBA	3	16	14	-	-	-	-	-	4-6
CRBA	2	16	9	5	1	-	-	-	4-8
CHBA	1	2	12	12	3	1	2	-	4-10
CBBA	-	8	11	6	6	2	-	-	5-9
CHuBA A^+	1	13	14	2	3	-	-	-	4-8
CHuBA A^-	2	13	12	4	2	-	-	-	4-8
CHuBA B^+	1	7	16	6	2	1	-	-	4-9
CHuBA B^-	-	5	9	17	1	1	-	-	5-9
CHuBA AB^+	1	16	10	5	1	-	-	-	4-8
CHuBA AB^-	3	10	15	4	-	1	-	-	4-9
CHuBA O^+	1	9	16	6	1	-	-	-	4-8
CHuBA O^-	4	12	14	2	1	-	-	-	4-8
[A] *Taxon C* (7 isolates / 6 strains [ETs])									
CAB	-	-	4	1	-	2	-	-	6-9
DSBA commercial	-	5	2	-	-	-	-	-	5-6
CSBA	3	3	-	1	-	-	-	-	4-7
CRBA	1	3	2	1	-	-	-	-	4-7
CHBA	-	2	3	1	1	-	-	-	5-8
CBBA	1	3	2	-	-	-	1	-	4-10
CHuBA A^+	-	3	3	-	1	-	-	-	5-8
CHuBA A^-	-	3	4	-	-	-	-	-	5-6
CHuBA B^+	2	3	1	-	1	-	-	-	4-8
CHuBA B^-	-	4	2	-	1	-	-	-	5-8
CHuBA AB^+	-	3	3	-	1	-	-	-	5-8
CHuBA AB^-	-	3	3	-	1	-	-	-	5-8
CHuBA O^+	1	3	2	-	1	-	-	-	4-8
CHuBA O^-	-	5	1	1	-	-	-	-	5-7

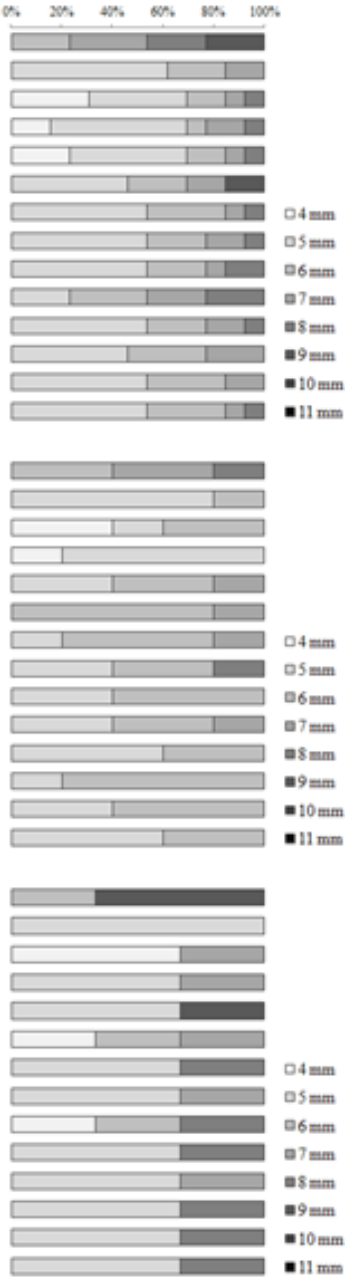


The letters [A] and [B] correspond to the Tukey grouping. The graphic to the right corresponds to the data from Table 2.

5. *Cluster* VII comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, III, IV, V, IX, X, XI, XII, XIII and XIV;
6. *Cluster* II comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* IV, V, VI, VIII, XI, XIII, XIV and XV;
7. *Cluster* III comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* IV, V, VI, VII, VIII, XI, XIII, XIV and XV;

Table 3. Profiles of the diameters of colonies within and among clusters moderately related and/or distantly genetically related (*clusters* of I to XV) of oxacillin-resistant *S. aureus* on thirteen different types of blood agar plates.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[E,F] *Cluster I* (13 isolates / 11 strains [ETs])									
CAB	-	-	3	4	3	3	-	-	6-9
DSBA commercial	-	8	3	2	-	-	-	-	5-7
CSBA	4	5	2	1	1	-	-	-	4-8
CRBA	2	7	1	2	1	-	-	-	4-8
CHBA	3	6	2	1	1	-	-	-	4-8
CBBA	-	6	3	2	-	2	-	-	4-7, 9
CHuBA A^+	-	7	4	1	1	-	-	-	5-8
CHuBA A^-	-	7	3	2	1	-	-	-	5-8
CHuBA B^+	-	7	3	1	2	-	-	-	5-8
CHuBA B^-	-	3	4	3	3	-	-	-	5-8
CHuBA AB^+	-	7	3	2	1	-	-	-	5-8
CHuBA AB^-	-	6	4	3	-	-	-	-	5-7
CHuBA O^+	-	7	4	2	-	-	-	-	5-7
CHuBA O^-	-	7	4	1	1	-	-	-	5-8
[D,E] *Cluster II* (5 isolates / 2 strains [ETs])									
CAB	-	-	2	2	1	-	-	-	6-8
DSBA commercial	-	4	1	-	-	-	-	-	5-6
CSBA	2	1	2	-	-	-	-	-	4-6
CRBA	1	4	-	-	-	-	-	-	4-5
CHBA	-	2	2	1	-	-	-	-	5-7
CBBA	-	-	4	1	-	-	-	-	6-7
CHuBA A^+	-	1	3	1	-	-	-	-	5-7
CHuBA A^-	-	2	2	-	1	-	-	-	5-6, 8
CHuBA B^+	-	2	3	-	-	-	-	-	5-6
CHuBA B^-	-	2	2	1	-	-	-	-	5-7
CHuBA AB^+	-	3	2	-	-	-	-	-	5-6
CHuBA AB^-	-	1	4	-	-	-	-	-	5-6
CHuBA O^+	-	2	3	-	-	-	-	-	5-6
CHuBA O^-	-	3	2	-	-	-	-	-	5-6
[E] *Cluster III* (3 isolates / 3 strains [ETs])									
CAB	-	-	1	-	-	2	-	-	6, 9
DSBA commercial	-	3	-	-	-	-	-	-	5
CSBA	2	-	-	1	-	-	-	-	4, 7
CRBA	-	2	-	1	-	-	-	-	5, 7
CHBA	-	2	-	-	-	1	-	-	5, 9
CBBA	1	-	1	1	-	-	-	-	4, 6-7
CHuBA A^+	-	2	-	-	1	-	-	-	5, 8
CHuBA A^-	-	2	-	1	-	-	-	-	5, 7
CHuBA B^+	1	-	1	-	1	-	-	-	4, 6, 8
CHuBA B^-	-	2	-	-	1	-	-	-	5, 8
CHuBA AB^+	-	2	-	1	-	-	-	-	5, 7
CHuBA AB^-	-	2	-	-	1	-	-	-	5, 8
CHuBA O^+	-	2	-	-	1	-	-	-	5, 8
CHuBA O^-	-	2	-	-	1	-	-	-	5, 8

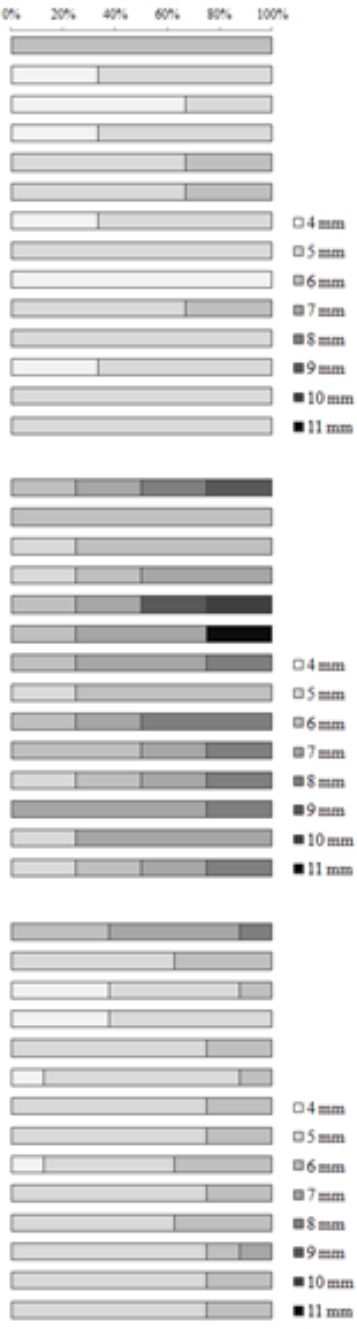


The letters A, B, C, D, E, F, G and H correspond to the Tukey grouping. The graphic to the right corresponds to the data from Table 3.

8. *Clusters* I, IX, X and XII comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* IV, V, VI, VII, VIII, XI, XIV and XV;
9. *Cluster* XIII comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* II, III, IV, V, VI, VII, VIII, XIV and XV; and
10. *Cluster* V comprised isolates/strains with variations in the diameter of bacterial colonies statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, IV, VI, VII, VIII, IX, X, XII, XIII, XIV and XV.

Table 3 Contd.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[A,B] *Cluster IV* (3 isolates / 3 strains [ETs])									
CAB	-	-	3	-	-	-	-	-	6
DSBA commercial	1	2	-	-	-	-	-	-	4-5
CSBA	2	1	-	-	-	-	-	-	4-5
CRBA	1	2	-	-	-	-	-	-	4-5
CHBA	-	2	1	-	-	-	-	-	5-6
CBBA	-	2	1	-	-	-	-	-	5-6
CHuBA A^+	1	2	-	-	-	-	-	-	4-5
CHuBA A^-	-	3	-	-	-	-	-	-	5
CHuBA B^+	3	-	-	-	-	-	-	-	4
CHuBA B^-	-	2	1	-	-	-	-	-	5-6
CHuBA AB^+	-	3	-	-	-	-	-	-	5
CHuBA AB^-	1	2	-	-	-	-	-	-	4-5
CHuBA O^+	-	3	-	-	-	-	-	-	5
CHuBA O^-	-	3	-	-	-	-	-	-	5
[H] *Cluster V* (4 isolates / 3 strains [ETs])									
CAB	-	-	1	1	1	1	-	-	6-9
DSBA commercial	-	-	4	-	-	-	-	-	6
CSBA	-	1	3	-	-	-	-	-	5-6
CRBA	-	1	1	2	-	-	-	-	5-7
CHBA	-	-	1	1	-	1	1	-	6-7, 9-10
CBBA	-	-	1	2	-	-	-	1	6-7, 11
CHuBA A^+	-	-	1	2	1	-	-	-	6-8
CHuBA A^-	-	1	3	-	-	-	-	-	5-6
CHuBA B^+	-	-	1	1	2	-	-	-	6-8
CHuBA B^-	-	-	2	1	1	-	-	-	6-8
CHuBA AB^+	-	1	1	1	1	-	-	-	5-8
CHuBA AB^-	-	-	-	3	1	-	-	-	7-8
CHuBA O^+	-	1	-	3	-	-	-	-	5, 7
CHuBA O^-	-	1	1	1	1	-	-	-	5-8
[C] *Cluster VI* (8 isolates / 6 strains [ETs])									
CAB	-	-	3	4	1	-	-	-	6-8
DSBA commercial	-	5	3	-	-	-	-	-	5-6
CSBA	3	4	1	-	-	-	-	-	4-6
CRBA	3	5	-	-	-	-	-	-	4-5
CHBA	-	6	2	-	-	-	-	-	5-6
CBBA	1	6	1	-	-	-	-	-	4-6
CHuBA A^+	-	6	2	-	-	-	-	-	5-6
CHuBA A^-	-	6	2	-	-	-	-	-	5-6
CHuBA B^+	1	4	3	-	-	-	-	-	4-6
CHuBA B^-	-	6	2	-	-	-	-	-	5-6
CHuBA AB^+	-	5	3	-	-	-	-	-	5-6
CHuBA AB^-	-	6	1	1	-	-	-	-	5-7
CHuBA O^+	-	6	2	-	-	-	-	-	5-6
CHuBA O^-	-	6	2	-	-	-	-	-	5-6

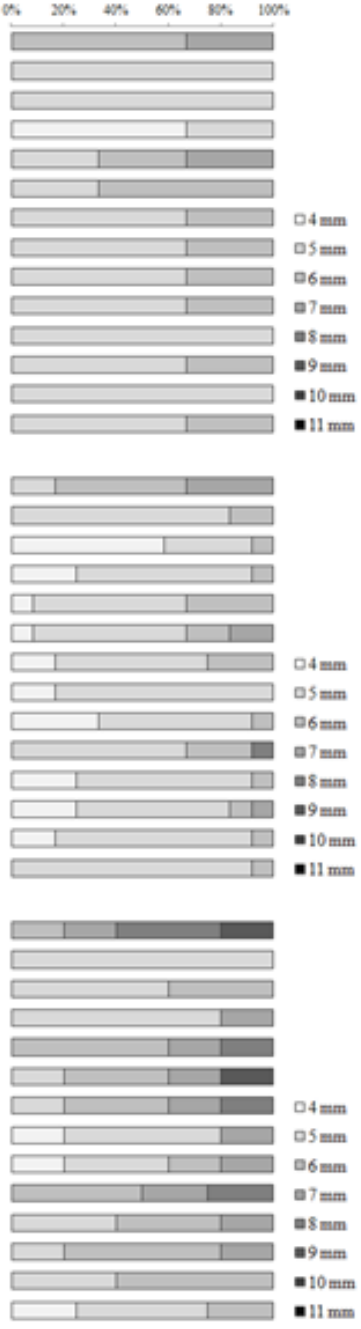


The frequency of bacterial isolates capable of expressing hemolysins *in vitro* varied quantitatively and qualitatively (Pz: indexes 1 and 2 for obvious or faint and index 0 for absent) depending on the BA culture media tested. The results indicated that the CRBA culture medium allowed the identification of a large number of isolates capable of expressing hemolysin (74% of the bacterial population), followed by CHuBA (53-63% of bacterial population), CSBA (48% of bacterial population), CHBA (35% of bacterial population) and CBBA (1% of bacterial population).

Surprisingly, the *S. aureus* isolates were unable to produce any hemolytic activity *in vitro* when using DSBA commercial culture medium. However, significant

Table 3 Contd.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[C,D] *Cluster VII* (3 isolates / 3 strains [ETs])									
CAB	-	-	2	1	-	-	-	-	6-7
DSBA commercial	-	3	-	-	-	-	-	-	5
CSBA	-	3	-	-	-	-	-	-	5
CRBA	2	1	-	-	-	-	-	-	4-5
CHBA	-	1	1	1	-	-	-	-	5-7
CBBA	-	1	2	-	-	-	-	-	5-6
CHuBA A^+	-	2	1	-	-	-	-	-	5-6
CHuBA A^-	-	2	1	-	-	-	-	-	5-6
CHuBA B^+	-	2	1	-	-	-	-	-	5-6
CHuBA B^-	-	2	1	-	-	-	-	-	5-6
CHuBA AB^+	-	3	-	-	-	-	-	-	5
CHuBA AB^-	-	2	1	-	-	-	-	-	5-6
CHuBA O^+	-	3	-	-	-	-	-	-	5
CHuBA O^-	-	2	1	-	-	-	-	-	5-6
[B,C] *Cluster VIII* (12 isolates / 3 strains [ETs])									
CAB	-	2	6	4	-	-	-	-	5-7
DSBA commercial	-	10	2	-	-	-	-	-	5-6
CSBA	7	4	1	-	-	-	-	-	4-6
CRBA	3	8	1	-	-	-	-	-	4-6
CHBA	1	7	4	-	-	-	-	-	4-6
CBBA	1	7	2	2	-	-	-	-	4-7
CHuBA A^+	2	7	3	-	-	-	-	-	4-6
CHuBA A^-	2	10	-	-	-	-	-	-	4-5
CHuBA B^+	4	7	1	-	-	-	-	-	4-6
CHuBA B^-	-	8	3	-	1	-	-	-	5-6, 8
CHuBA AB^+	3	8	1	-	-	-	-	-	4-6
CHuBA AB^-	3	7	1	1	-	-	-	-	4-7
CHuBA O^+	2	9	1	-	-	-	-	-	4-6
CHuBA O^-	-	11	1	-	-	-	-	-	5-6
[E,F] *Cluster IX* (5 isolates / 4 strains [ETs])									
CAB	-	-	1	1	2	1	-	-	6-9
DSBA commercial	-	5	-	-	-	-	-	-	5
CSBA	-	3	2	-	-	-	-	-	5-6
CRBA	-	4	-	1	-	-	-	-	5,7
CHBA	-	-	3	1	1	-	-	-	6-8
CBBA	-	1	2	1	-	1	-	-	5-7, 9
CHuBA A^+	-	1	2	1	1	-	-	-	5-8
CHuBA A^-	1	3	-	1	-	-	-	-	4-5, 7
CHuBA B^+	1	2	1	1	-	-	-	-	4-7
CHuBA B^-	-	-	2	1	1	-	-	-	6-8
CHuBA AB^+	-	2	2	1	-	-	-	-	5-7
CHuBA AB^-	-	1	3	1	-	-	-	-	5-7
CHuBA O^+	-	2	3	-	-	-	-	-	5-6
CHuBA O^-	1	2	1	-	-	-	-	-	4-6

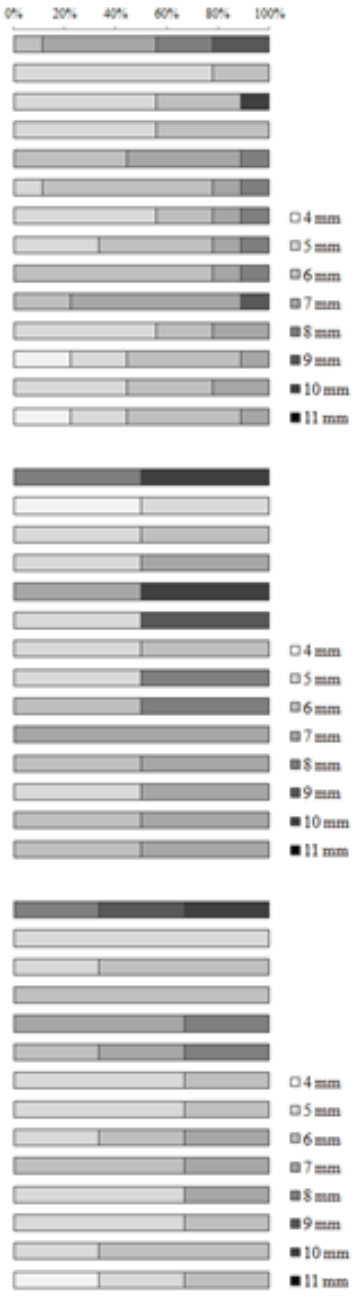


($p < 0.05$) were observed among the BA media in five cases (Table 4):

1. CBBA and DSBA commercial provided the expression of the hemolytic activity for *S. aureus* isolates statistically different ($p < 0.05$) when compared to CHBA, CSBA, CRBA, CHuBA A^-, CHuBA A^+, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^- and CHuBA O^+;
2. CHBA provided the expression of the hemolytic activity for *S. aureus* isolates statistically different ($p < 0.05$) when compared to CBBA, CSBA, DSBA commercial, CRBA, CHuBA A^-, CHuBA A^+, CHuBA AB^-, CHuBA AB^+,

Table 3. Contd.

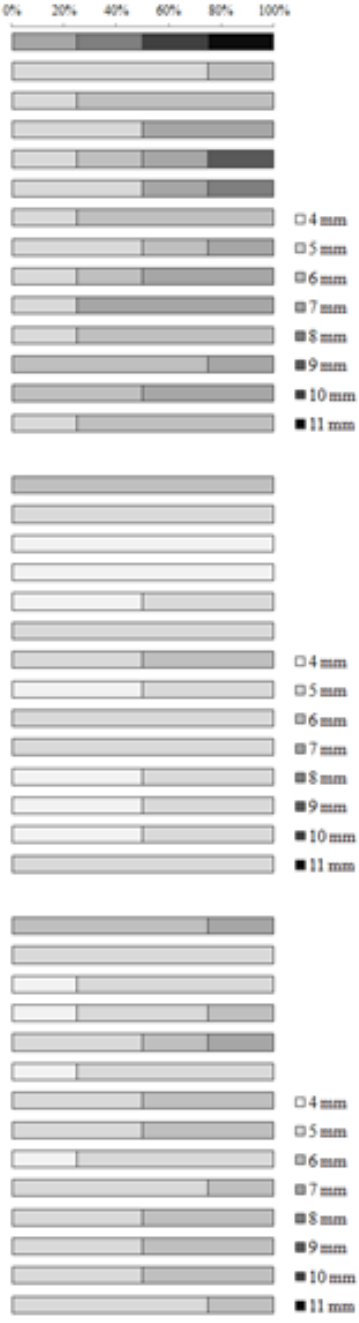
Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[E,F] *Cluster X* (9 isolates / 9 strains [ETs])									
CAB	-	-	1	4	2	2	-	-	6-9
DSBA commercial	-	7	2	-	-	-	-	-	5-6
CSBA	-	5	3	-	-	-	1	-	5-6, 10
CRBA	-	5	4	-	-	-	-	-	5-6
CHBA	-	-	4	4	1	-	-	-	6-8
CBBA	-	1	6	1	1	-	-	-	5-8
CHuBA A^+	-	5	2	1	1	-	-	-	5-8
CHuBA A^-	-	3	4	1	1	-	-	-	5-8
CHuBA B^+	-	-	7	1	1	-	-	-	6-8
CHuBA B^-	-	-	2	6	-	1	-	-	6-7, 9
CHuBA AB^+	-	5	2	2	-	-	-	-	5-7
CHuBA AB^-	2	2	4	1	-	-	-	-	4-7
CHuBA O^+	-	4	3	2	-	-	-	-	5-7
CHuBA O^-	2	2	4	1	-	-	-	-	4-7
[G,H] *Cluster XI* (2 isolates / 2 strains [ETs])									
CAB	-	-	-	-	1	-	1	-	8, 10
DSBA commercial	1	1	-	-	-	-	-	-	4-5
CSBA	-	1	1	-	-	-	-	-	5-6
CRBA	-	1	-	1	-	-	-	-	5, 7
CHBA	-	-	-	1	-	-	1	-	7, 10
CBBA	-	1	-	-	-	1	-	-	5, 9
CHuBA A^+	-	1	1	-	-	-	-	-	5-6
CHuBA A^-	-	1	-	-	1	-	-	-	5, 8
CHuBA B^+	-	-	1	-	1	-	-	-	6, 8
CHuBA B^-	-	-	-	2	-	-	-	-	7
CHuBA AB^+	-	-	1	1	-	-	-	-	6-7
CHuBA AB^-	-	1	-	1	-	-	-	-	5, 7
CHuBA O^+	-	-	1	1	-	-	-	-	6-7
CHuBA O^-	-	-	1	1	-	-	-	-	6-7
[E,F] *Cluster XII* (3 isolates / 3 strains [ETs])									
CAB	-	-	-	-	1	1	1	-	8-10
DSBA commercial	-	3	-	-	-	-	-	-	5
CSBA	-	1	2	-	-	-	-	-	5-6
CRBA	-	-	3	-	-	-	-	-	6
CHBA	-	-	-	2	1	-	-	-	7-8
CBBA	-	-	1	1	1	-	-	-	6-8
CHuBA A^+	-	2	1	-	-	-	-	-	5-6
CHuBA A^-	-	2	1	-	-	-	-	-	5-6
CHuBA B^+	-	1	1	1	-	-	-	-	5-7
CHuBA B^-	-	-	2	1	-	-	-	-	6-7
CHuBA AB^+	-	2	-	1	-	-	-	-	5, 7
CHuBA AB^-	-	2	1	-	-	-	-	-	5-6
CHuBA O^+	-	1	2	-	-	-	-	-	5-6
CHuBA O^-	1	1	1	-	-	-	-	-	4-6



CHuBA B^-, CHuBA B^+, CHuBA O^- and CHuBA O^+;
3. CSBA, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^- and CHuBA O^+ provided the expression of the hemolytic activity for *S. aureus* isolates statistically different ($p < 0.05$) when compared to CBBA, CHBA, DSBA commercial, CRBA, CHuBA A^- and CHuBA A^+;
4. CHuBA A^-, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^-, CHuBA O^+, CHuBA A^+ provided the expression of the hemolytic activity for *S. aureus* isolates statistically different ($p < 0.05$) when compared to CBBA, DSBA commercial, CHBA, CSBA and CRBA; and
5. CRBA and CHuBA A^+ provided the expression of the hemolytic activity for *S. aureus* isolates statistically different ($p < 0.05$) when compared to CBBA, CHBA, CSBA, DSBA commercial, CHuBA A^-, CHuBA AB^-, CHuBA AB^+, CHuBA B^-, CHuBA B^+, CHuBA O^- and

Table 3. Contd.

Culture media	Number of isolates (*n* mm of ∅)								Range
	4 mm	5 mm	6 mm	7 mm	8 mm	9 mm	10 mm	11 mm	mm (∅)
[F,G] *Cluster XIII* (4 isolates / 2 strains [ETs])									
CAB	-	-	-	1	1	-	1	1	7-8, 10-11
DSBA commercial	-	3	1	-	-	-	-	-	5-6
CSBA	-	1	3	-	-	-	-	-	5-6
CRBA	-	2	-	2	-	-	-	-	5, 7
CHBA	-	1	1	1	-	1	-	-	5-7, 9
CBBA	-	2	-	1	1	-	-	-	5, 7-8
CHuBA A^+	-	1	3	-	-	-	-	-	5-6
CHuBA A^-	-	2	1	1	-	-	-	-	5-7
CHuBA B^+	-	1	1	2	-	-	-	-	5-7
CHuBA B^-	-	1	-	3	-	-	-	-	5, 7
CHuBA AB^+	-	1	3	-	-	-	-	-	5-6
CHuBA AB^-	-	-	3	1	-	-	-	-	6-7
CHuBA O^+	-	-	2	2	-	-	-	-	6-7
CHuBA O^-	-	1	3	-	-	-	-	-	5-6
[A] *Cluster XIV* (2 isolates / 2 strains [ETs])									
CAB	-	-	2	-	-	-	-	-	6
DSBA commercial	-	2	-	-	-	-	-	-	5
CSBA	2	-	-	-	-	-	-	-	4
CRBA	2	-	-	-	-	-	-	-	4
CHBA	1	1	-	-	-	-	-	-	4-5
CBBA	-	2	-	-	-	-	-	-	5
CHuBA A^+	-	1	1	-	-	-	-	-	5-6
CHuBA A^-	1	1	-	-	-	-	-	-	4-5
CHuBA B^+	-	2	-	-	-	-	-	-	5
CHuBA B^-	-	2	-	-	-	-	-	-	5
CHuBA AB^+	1	1	-	-	-	-	-	-	4-5
CHuBA AB^-	1	1	-	-	-	-	-	-	4-5
CHuBA O^+	1	1	-	-	-	-	-	-	4-5
CHuBA O^-	-	2	-	-	-	-	-	-	5
[C] *Cluster XV* (4 isolates / 3 strains [ETs])									
CAB	-	-	3	1	-	-	-	-	6-7
DSBA commercial	-	4	-	-	-	-	-	-	5
CSBA	1	3	-	-	-	-	-	-	4-5
CRBA	1	2	1	-	-	-	-	-	4-6
CHBA	-	2	1	1	-	-	-	-	5-7
CBBA	1	3	-	-	-	-	-	-	4-5
CHuBA A^+	-	2	2	-	-	-	-	-	5-6
CHuBA A^-	-	2	2	-	-	-	-	-	5-6
CHuBA B^+	1	3	-	-	-	-	-	-	4-5
CHuBA B^-	-	3	1	-	-	-	-	-	5-6
CHuBA AB^+	-	2	2	-	-	-	-	-	5-6
CHuBA AB^-	-	2	2	-	-	-	-	-	5-6
CHuBA O^+	-	2	2	-	-	-	-	-	5-6
CHuBA O^-	-	3	1	-	-	-	-	-	5-6

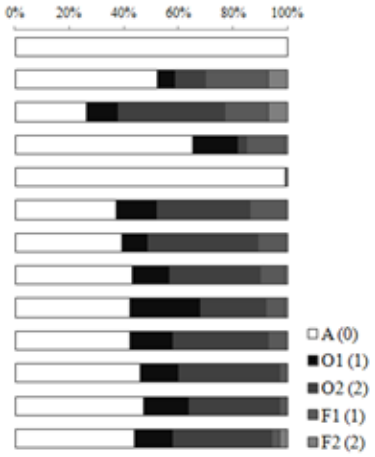


CHuBA O^+.

The hemolysis *in vitro* activities were also evaluated within and between the major taxonomic ranks of ORSA [that is, *taxa* A (60 isolates/43 strains), B (33 isolates/30 strains) and C (7 isolates/6 strains). The profiles of the hemolytic activities revealed significant differences ($p < 0.05$) between *taxa* A and C, as well as B and C. *Taxa* A and B were considered statistically identical to the hemolysis profiles produced by ORSA based on their ranks on each type of BA medium (Table 5). The *in vitro* hemolytic profiles evaluated within and among the lowest taxonomic ranks of ORSA (that is, from *clusters* I to XV) revealed significant differences ($p < 0.05$) between

Table 4. Percent index of hemolysis activity (*Pz*) of oxacillin-resistant *S. aureus* [99 isolates (79 strains/ETs) and reference strain ATCC® 25923™] on 13 different types of blood agar plates.

Culture media	Hemolysis activity (*Pz*)*				
	Absent	Obvious		Faint	
	A (0)	O1 (1)	O2 (2)	F1 (1)	F2 (2)
[A] DSBA commercial	100	-	-	-	-
[C] CSBA	52	7	11	23	7
[E] CRBA	26	12	39	16	7
[B] CHBA	65	17	3	14	1
[A] CBBA	99	-	-	1	-
[D, E] CHuBA A+	37	15	34	14	-
[D] CHuBA A-	39	10	40	11	-
[D, C] CHuBA B+	43	14	33	9	1
[D, C] CHuBA B-	42	26	24	7	1
[D, C] CHuBA AB+	42	16	35	7	-
[D, C] CHuBA AB-	46	14	37	3	-
[D, C] CHuBA O+	47	17	33	3	-
[D, C] CHuBA O-	44	14	36	3	3



**Pz* indexes equal to 0 $^{Pz = 1}$, 1 $^{0.64 < Pz < 1}$ and 2 $^{Pz < 0.64}$ correspond to absent, positive and strongly positive enzyme activity, respectively. The graphic to the right corresponds to the data from Table 4. The letters [A], [B], [C], [D] and [E] correspond to the Tukey grouping.

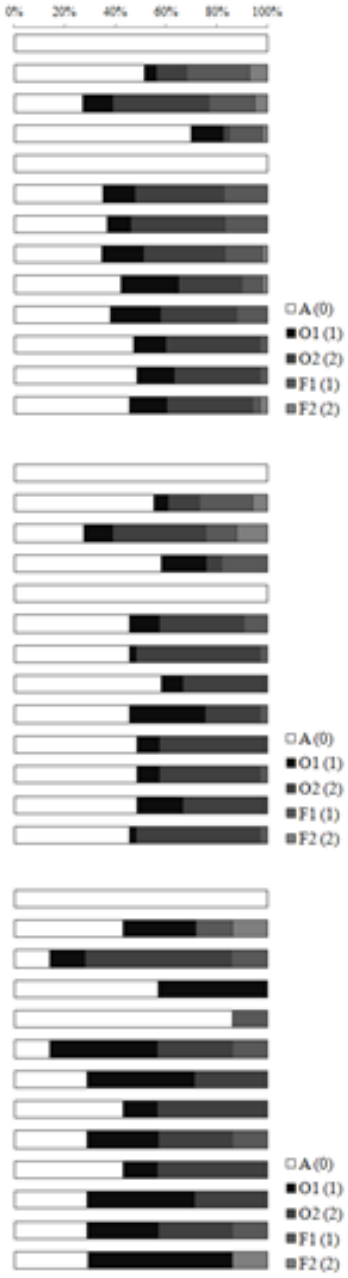
clusters in six cases (Table 6):
1. *Cluster* IV comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XIII, XIV and XV;
2. *Clusters* VII, VIII and IX comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* I, III, IV, V, VI, XII and XIV;
3. *Clusters* XI, XIII and XV comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* III, IV and XIV;
4. *Clusters* II and X comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* III, IV, XII and XIV;
5. *Clusters* I, V and VI comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* III, IV, VII, VIII, IX, XII and XIV; and
6. *Clusters* III and XIV comprised isolates/strains able to express hemolytic activity statistically different ($p < 0.05$) from those observed in *clusters* I, II, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII and XV.

The morphological aspects of the bacterial colonies of all ORSA isolates, including the reference strain ATCC® 25923™, were 99% shiny *versus* 1% opaque, 53% yellow *versus* 47% white and 98% glossy versus 2% dry, regardless of the type of BA culture media (Table 7). In each taxonomic *rank* of ORSA isolates (major ranks: A, B and C *taxa*; minor ranks: *clusters* I to XV), these morphological aspects were observed regardless of the BA media type, although with intrinsic characteristics for each rank. Significant differences ($p < 0.05$) were observed between the *taxa* A and B or A and C in terms of shiny/opaque and yellow/white aspects, and, in addition, there were differences between the *taxa* B and C regarding the glossy/dry aspect (Table 8). No significant difference ($p < 0.05$) was observed between the *clusters* regarding shiny/opaque colonies. For glossy/dry, differences were observable between *clusters* in only one situation [that is, cluster X comprised a significant percentage of isolates/strains exhibiting morphological aspects of dry bacterial colonies (11.1%) when compared to other *clusters* (0.0%)]. For the yellow/white aspect, such differences were observed between *clusters* in nine situations (Table 9):

1. *Cluster* VII comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* I, II, III, IV, V, VI, IX, X, XI, XII, XIII, XIV and XV;
2. *Cluster* VIII comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* I, IV, V, VI, IX, X, XI, XII, XIII, XIV and XV;
3. *Cluster* II comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* I, IV, V, VI, VII, IX, X, XI, XII, XIII, XIV and XV;
4. *Cluster* III comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* IV, V, VII, X, XII and XIII;

Table 5. Percent index of hemolysis activity (*Pz*) within and among distantly genetically related populations (*taxa* A, B and C) of oxacillin-resistant *S. aureus* on thirteen different types of blood agar plates.

Culture media	Hemolysis activity (*Pz*)*				
	Absent	Obvious		Faint	
	A (0)	O1 (1)	O2 (2)	F1 (1)	F2 (2)
[A] *Taxon A* (60 isolates / 43 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	52	5	12	25	7
CRBA	27	12	38	18	5
CHBA	70	13	2	13	2
CBBA	100	-	-	-	-
CHuBA A^+	35	13	35	17	-
CHuBA A^-	37	10	37	17	-
CHuBA B^+	35	17	32	15	2
CHuBA B^-	42	23	25	8	2
CHuBA AB^+	38	20	30	12	-
CHuBA AB^-	47	13	37	3	-
CHuBA O^+	48	15	33	3	-
CHuBA O^-	45	15	33	3	3
[A] *Taxon B* (33 isolates / 30 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	55	6	12	21	6
CRBA	27	12	36	12	12
CHBA	58	18	6	18	-
CBBA	100	-	-	-	-
CHuBA A^+	45	12	33	9	-
CHuBA A^-	45	3	48	3	-
CHuBA B^+	58	9	33	-	-
CHuBA B^-	45	30	21	3	-
CHuBA AB^+	48	9	42	-	-
CHuBA AB^-	48	9	39	3	-
CHuBA O^+	48	18	33	-	-
CHuBA O^-	45	3	48	3	-
[B] *Taxon C* (7 isolates / 6 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	43	29	-	14	14
CRBA	14	14	57	14	-
CHBA	57	43	-	-	-
CBBA	86	-	-	14	-
CHuBA A^+	14	43	29	14	-
CHuBA A^-	29	43	29	-	-
CHuBA B^+	43	14	43	-	-
CHuBA B^-	29	29	29	14	-
CHuBA AB^+	43	14	43	-	-
CHuBA AB^-	29	43	29	-	-
CHuBA O^+	29	29	29	14	-
CHuBA O^-	29	57	-	-	14



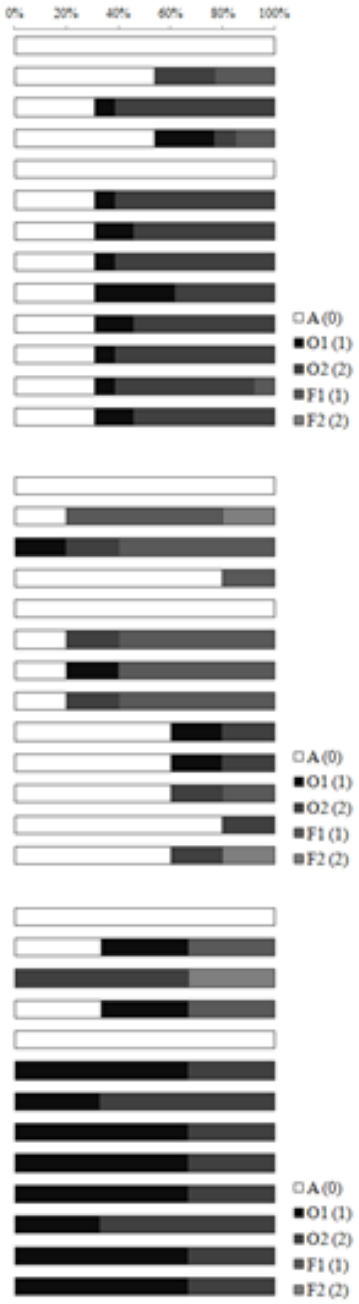
* Pz indexes equal to 0 $^{Pz = 1}$, 1 $^{0.64 < Pz < 1}$ and 2 $^{Pz < 0.64}$ correspond to absent, positive and strongly positive enzyme activity, respectively. The three graphics to the right correspond to the data of each taxon from Table 5. The letters A and B correspond to the Tukey grouping.

5. *Clusters* I, VI and IX comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* II, IV, V, VII, VIII, X, XII and XIII;

6. *Clusters* XI, XIV and XV comprised a significant

Table 6. Percent index of hemolysis activity (*Pz*) within and among *clusters* moderately related and/or distantly genetically related (*clusters* of I to XV) of oxacillin-resistant *S. aureus* on thirteen different types of blood agar plates.

Culture media	Hemolysis activity (*Pz*)*				
	Absent	Obvious		Faint	
	A (0)	O1 (1)	O2 (2)	F1 (1)	F2 (2)
[D] *Cluster I* (13 isolates / 11 strains [ETx])					
DSBA commercial	100	-	-	-	-
CSBA	54	-	23	23	-
CRBA	31	8	62	-	-
CHBA	54	23	8	15	-
CBBA	100	-	-	-	-
CHuBA A^+	31	8	62	-	-
CHuBA A^-	31	15	54	-	-
CHuBA B^+	31	8	62	-	-
CHuBA B^-	31	31	38	-	-
CHuBA AB^+	31	15	54	-	-
CHuBA AB^-	31	8	62	-	-
CHuBA O^+	31	8	54	8	-
CHuBA O^-	31	15	54	-	-
[C, D] *Cluster II* (5 isolates / 2 strains [ETx])					
DSBA commercial	100	-	-	-	-
CSBA	20	-	-	60	20
CRBA	-	20	20	60	-
CHBA	80	-	-	20	-
CBBA	100	-	-	-	-
CHuBA A^+	20	-	20	60	-
CHuBA A^-	20	20	-	60	-
CHuBA B^+	20	-	20	60	-
CHuBA B^-	60	20	20	-	-
CHuBA AB^+	60	20	20	-	-
CHuBA AB^-	60	-	20	20	-
CHuBA O^+	80	-	20	-	-
CHuBA O^-	60	-	20	-	20
[E] *Cluster III* (3 isolates / 3 strains [ETx])					
DSBA commercial	100	-	-	-	-
CSBA	33	33	-	33	-
CRBA	-	-	67	-	33
CHBA	33	33	-	33	-
CBBA	100	-	-	-	-
CHuBA A^+	-	67	33	-	-
CHuBA A^-	-	33	67	-	-
CHuBA B^+	-	67	33	-	-
CHuBA B^-	-	67	33	-	-
CHuBA AB^+	-	67	33	-	-
CHuBA AB^-	-	33	67	-	-
CHuBA O^+	-	67	33	-	-
CHuBA O^-	-	67	33	-	-



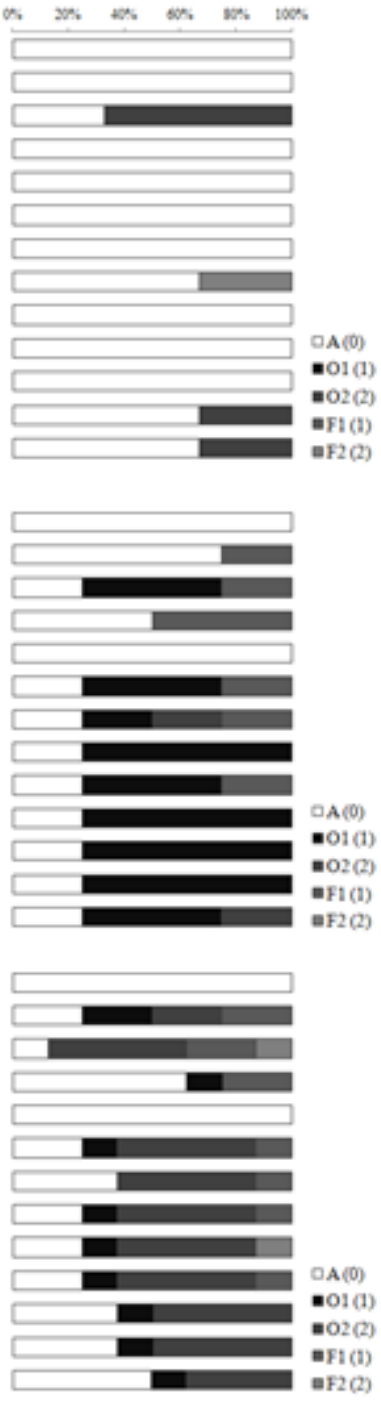
*Pz indexes equal to 0 $^{Pz = 1}$, 1 $^{0.64 < Pz < 1}$ and 2 $^{Pz < 0.64}$ correspond to absent, positive and strongly positive enzyme activity, respectively. The three graphics to the right correspond to the data of each cluster from Table 6, respectively. The letters A, B, C, D and E correspond to Tukey grouping.

percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* II, V, VII, VIII and XIII;

7. *Clusters* IV, X and XII comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when

Table 6, Contd.

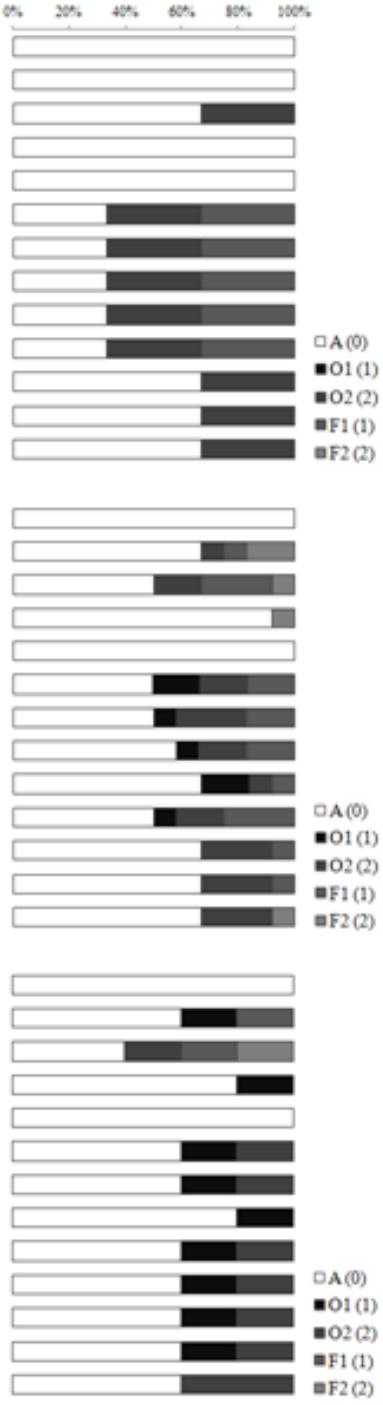
Culture media	Hemolysis activity (*Pz*)*				
	Absent	Obvious		Faint	
	A (0)	O1 (1)	O2 (2)	F1 (1)	F2 (2)
[A] *Cluster IV* (3 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	100	-	-	-	-
CRBA	33	-	67	-	-
CHBA	100	-	-	-	-
CBBA	100	-	-	-	-
CHuBA A^+	100	-	-	-	-
CHuBA A^-	100	-	-	-	-
CHuBA B^+	67	-	-	-	33
CHuBA B^-	100	-	-	-	-
CHuBA AB^+	100	-	-	-	-
CHuBA AB^-	100	-	-	-	-
CHuBA O^+	67	-	33	-	-
CHuBA O^-	67	-	33	-	-
[D] *Cluster V* (4 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	75	-	-	25	-
CRBA	25	50	-	25	-
CHBA	50	-	-	50	-
CBBA	100	-	-	-	-
CHuBA A^+	25	50	-	25	-
CHuBA A^-	25	25	25	25	-
CHuBA B^+	25	75	-	-	-
CHuBA B^-	25	50	-	25	-
CHuBA AB^+	25	75	-	-	-
CHuBA AB^-	25	75	-	-	-
CHuBA O^+	25	75	-	-	-
CHuBA O^-	25	50	25	-	-
[D] *Cluster VI* (8 isolates / 6 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	25	25	25	25	-
CRBA	13	-	50	25	13
CHBA	63	13	-	25	-
CBBA	100	-	-	-	-
CHuBA A^+	25	13	50	13	-
CHuBA A^-	38	-	50	13	-
CHuBA B^+	25	13	50	13	-
CHuBA B^-	25	13	50	-	13
CHuBA AB^+	25	13	50	13	-
CHuBA AB^-	38	13	50	-	-
CHuBA O^+	38	13	50	-	-
CHuBA O^-	50	13	38	-	-



compared to *clusters* I, II, III, V, VI, VII, VIII and IX;
8. *Cluster* XIII comprised a significant percentage ($p < 0.05$) of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to *clusters* I, II, III, V, VI, VII, VIII, IX, XI, XIV and XV; and
9. *Cluster* V comprised a significant percentage ($p < 0.05$)

Table 6. Contd.

Culture media	Hemolysis activity (*Pz*)* Absent A (0)	Obvious O1 (1)	Obvious O2 (2)	Faint F1 (1)	Faint F2 (2)
[B, C] *Cluster VII* (3 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	100	-	-	-	-
CRBA	67	-	33	-	-
CHBA	100	-	-	-	-
CBBA	100	-	-	-	-
CHuBA A^+	33	-	33	33	-
CHuBA A^-	33	-	33	33	-
CHuBA B^+	33	-	33	33	-
CHuBA B^-	33	-	33	33	-
CHuBA AB^+	33	-	33	33	-
CHuBA AB^-	67	-	33	-	-
CHuBA O^+	67	-	33	-	-
CHuBA O^-	67	-	33	-	-
[B, C] *Cluster VIII* (12 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	67	-	8	8	17
CRBA	50	-	17	25	8
CHBA	92	-	-	-	8
CBBA	100	-	-	-	-
CHuBA A^+	50	17	17	17	-
CHuBA A^-	50	8	25	17	-
CHuBA B^+	58	8	17	17	-
CHuBA B^-	67	17	8	8	-
CHuBA AB^+	50	8	17	25	-
CHuBA AB^-	67	-	25	8	-
CHuBA O^+	67	-	25	8	-
CHuBA O^-	67	-	25	-	8
[B, C] *Cluster IX* (5 isolates / 4 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	60	20	-	20	-
CRBA	40	-	20	20	20
CHBA	80	20	-	-	-
CBBA	100	-	-	-	-
CHuBA A^+	60	20	20	-	-
CHuBA A^-	60	20	20	-	-
CHuBA B^+	80	20	-	-	-
CHuBA B^-	60	20	20	-	-
CHuBA AB^+	60	20	20	-	-
CHuBA AB^-	60	20	20	-	-
CHuBA O^+	60	20	20	-	-
CHuBA O^-	60	-	40	-	-



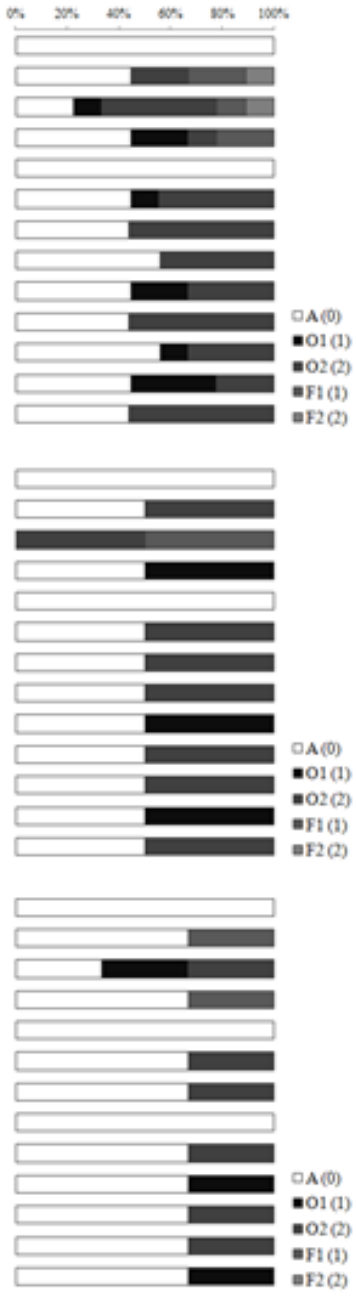
of isolates/strains exhibiting morphological aspects of yellow/white bacterial colonies when compared to other clusters.

DISCUSSION

The use of BA culture media has been demonstrated to

Table 6. Contd.

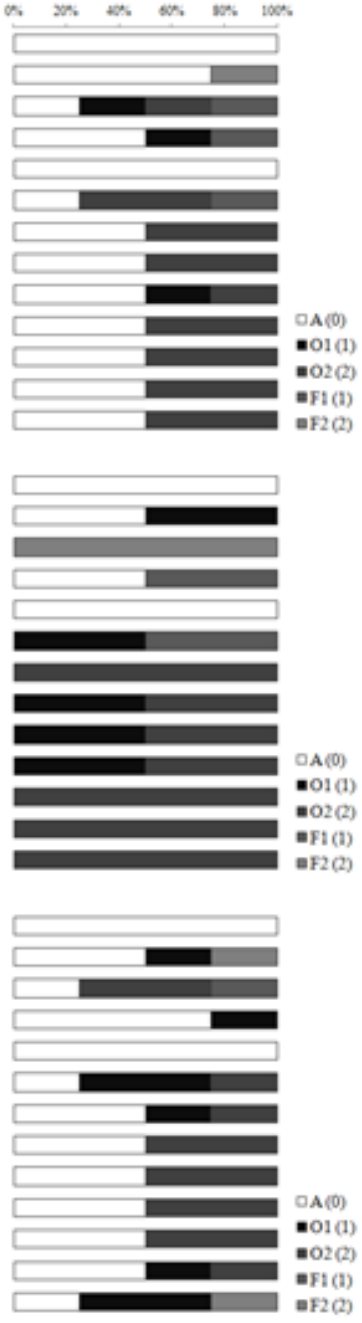
Culture media	Hemolysis activity (*Pz*)* Absent A (0)	Obvious O1 (1)	Obvious O2 (2)	Faint F1 (1)	Faint F2 (2)
[C, D] *Cluster X* (9 isolates / 9 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	44	-	22	22	11
CRBA	22	11	44	11	11
CHBA	44	22	11	22	-
CBBA	100	-	-	-	-
CHuBA A^+	44	11	44	-	-
CHuBA A^-	44	-	56	-	-
CHuBA B^+	56	-	44	-	-
CHuBA B^-	44	22	33	-	-
CHuBA AB^+	44	-	56	-	-
CHuBA AB^-	56	11	33	-	-
CHuBA O^+	44	33	22	-	-
CHuBA O^-	44	-	56	-	-
[B, C, D] *Cluster XI* (2 isolates / 2 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	50	-	50	-	-
CRBA	-	-	50	50	-
CHBA	50	50	-	-	-
CBBA	100	-	-	-	-
CHuBA A^+	50	-	50	-	-
CHuBA A^-	50	-	50	-	-
CHuBA B^+	50	-	50	-	-
CHuBA B^-	50	50	-	-	-
CHuBA AB^+	50	-	50	-	-
CHuBA AB^-	50	-	50	-	-
CHuBA O^+	50	50	-	-	-
CHuBA O^-	50	-	50	-	-
[A, B] *Cluster XII* (3 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	67	-	-	33	-
CRBA	33	33	33	-	-
CHBA	67	-	-	33	-
CBBA	100	-	-	-	-
CHuBA A^+	67	-	33	-	-
CHuBA A^-	67	-	33	-	-
CHuBA B^+	100	-	-	-	-
CHuBA B^-	67	-	33	-	-
CHuBA AB^+	67	33	-	-	-
CHuBA AB^-	67	-	33	-	-
CHuBA O^+	67	-	33	-	-
CHuBA O^-	67	33	-	-	-



be useful in clinical microbiological diagnosis of several bacterial infections, especially the species *S. aureus*, for the isolation and preliminary identification of these pathogens of medical importance and the subcultures that precede the phenotypic assays for identification and antimicrobial susceptibility (Anand et al., 2000; Egwuatu et al., 2014; Satzke et al., 2010; Sharp and Searcy, 2006) and the phenotypic characterization of certain virulence factors, such as the determination of exotoxins (α, β, δ and γ-hemolysins) (Ali-Vehmas et al., 2001; Bohach et al., 1997; Bohach and Foster, 2000; Peacok et al., 2002; Sakoulas et al., 2002) involved in the development of animal or human diseases (Ali-Vehmas et al., 2001; Yarwood and Schlievert, 2003). To the best of our

Table 6. Contd.

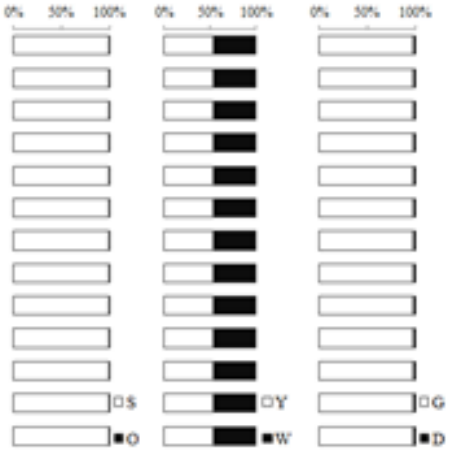
Culture media	Hemolysis activity (*Pz*)* Absent A (0)	Obvious O1 (1)	Obvious O2 (2)	Faint F1 (1)	Faint F2 (2)
[B, C, D] *Cluster XIII* (4 isolates / 2 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	75	-	-	-	25
CRBA	25	25	25	25	-
CHBA	50	25	-	25	-
CBBA	100	-	-	-	-
CHuBA A^+	25	-	50	25	-
CHuBA A^-	50	-	50	-	-
CHuBA B^+	50	-	50	-	-
CHuBA B^-	50	25	25	-	-
CHuBA AB^+	50	-	50	-	-
CHuBA AB^-	50	-	50	-	-
CHuBA O^+	50	-	50	-	-
CHuBA O^-	50	-	50	-	-
[E] *Cluster XIV* (2 isolates / 2 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	50	50	-	-	-
CRBA	-	-	-	-	100
CHBA	50	-	-	50	-
CBBA	100	-	-	-	-
CHuBA A^+	-	50	-	50	-
CHuBA A^-	-	-	100	-	-
CHuBA B^+	-	50	50	-	-
CHuBA B^-	-	50	50	-	-
CHuBA AB^+	-	50	50	-	-
CHuBA AB^-	-	-	100	-	-
CHuBA O^+	-	-	100	-	-
CHuBA O^-	-	-	100	-	-
[B, C, D] *Cluster XV* (4 isolates / 3 strains [ETs])					
DSBA commercial	100	-	-	-	-
CSBA	50	25	-	-	25
CRBA	25	-	50	25	-
CHBA	75	25	-	-	-
CBBA	100	-	-	-	-
CHuBA A^+	25	50	25	-	-
CHuBA A^-	50	25	25	-	-
CHuBA B^+	50	-	50	-	-
CHuBA B^-	50	-	50	-	-
CHuBA AB^+	50	-	50	-	-
CHuBA AB^-	50	-	50	-	-
CHuBA O^+	50	25	25	-	-
CHuBA O^-	25	50	-	-	25



knowledge, this is the first study to compare colonial morphology [size (millimeter of ∅) and appearance (shiny or opaque, yellow or white, glossy or dry)] and hemolysis activity (*Pz* obvious or faint) of oxacillin-resistant *S. aureus*, from odontological patients and clinical environmental (air) samples and characterized genetically in terms of population, subpopulations (*taxa*), *clusters* and strains [ETs] using 13 BA culture media with assays conducted in triplicate (DSBA commercial, CSBA, CBBA, CHBA, CRBA, CHuBA O^-, CHuBA O^+, CHuBA A^-, CHuBA A^+, CHuBA B^-, CHuBA B^+, CHuBA AB^- and CHuBA AB^+), including a control CAB culture medium. Phenotypic

Table 7. Percent index of the morphological features of bacterial colonies of oxacillin-resistant *S. aureus* [99 isolates (79 strains/ETs) and reference strain ATCC® 25923] on thirteen different types of blood agar plates.

Culture media	Morphology of colonies					
	S	O	Y	W	G	D
[A] DSBA commercial	99	1	53	47	98	2
[A] CSBA	99	1	53	47	98	2
[A] CRBA	99	1	53	47	98	2
[A] CHBA	99	1	53	47	98	2
[A] CBBA	99	1	53	47	98	2
[A] CHuBA A^+	99	1	53	47	98	2
[A] CHuBA A^-	99	1	53	47	98	2
[A] CHuBA B^+	99	1	53	47	98	2
[A] CHuBA B^-	99	1	53	47	98	2
[A] CHuBA AB^+	99	1	53	47	98	2
[A] CHuBA AB^-	99	1	53	47	98	2
[A] CHuBA O^+	99	1	53	47	98	2
[A] CHuBA O^-	99	1	53	47	98	2



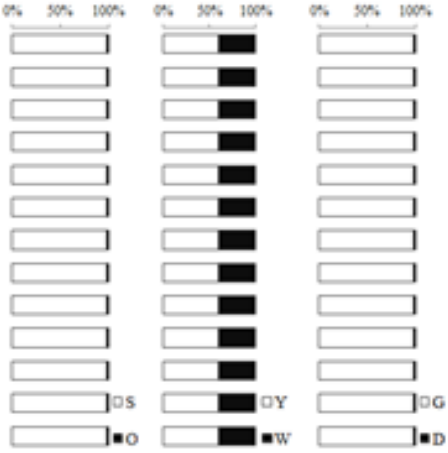
The letters S, O, Y, W, G and D correspond to shiny, opaque, yellow, white, glossy, and dry, respectively. The graphics to the right correspond to the data from Table 7. The letter [A] corresponds to Tukey grouping.

variability can be observed between different strains and even between different isolates belonging to the same strains (that is, variability in appearance and size of the colony and in the β-hemolytic activity depending on the BA culture media) (Supplemental Table 1). As for the size of the diameter of the colonies on these culture media, variations were observed (4-11 mm of ∅) in all the population of bacterial isolates, with 5-6 mm being the diameter range reached by most of these isolates. However, significant differences were observed between the BA culture media in seven distinct situations (Table 1), among *taxa* A and B or B and C (*taxa* A and C were considered statistically identical) (Table 2) and also among clusters in 10 distinct situations (Table 3). The frequency of bacterial isolates capable of expressing hemolysins *in vitro* varied both quantitatively and qualitatively (indexes *Pz*) depending on the BA culture media tested (Table 4). The results revealed that the CRBA culture medium facilitated the identification of a large number of isolates able to express hemolysins (74% of the bacterial population), followed by the CHuBA culture media (53-63%), CSBA (48%), CHBA (35%) and CBBA (1%).

Surprisingly, the *S. aureus* isolates were unable to produce any hemolytic activity *in vitro* on commercial DSBA culture medium. For this hemolytic activity, significant differences were observed among the BA culture media in five different situations (Table 4), between *taxa* A and C or B and C (the *taxa* A and B were considered statistically identical) (Table 5) and among clusters in six distinct situations (Table 6). These results suggest that the expression of hemolysins by oxacillin-resistant *S. aureus* can be favored by a bacterial intrinsic mechanism depending on the use of a particular BA culture media designated for bacteriological diagnostics; in addition, there is a deficit in colonial growth potential (for example, the CRBA culture medium). However, the expression of hemolysins by oxacillin-resistant *S. aureus* can be partially favored or blocked depending on the use of particular BA culture media, but this is associated with a greater potential for colonial growth (for example, the CHBA and CBBA culture media). In turn, the CHuBA and CSBA culture media appeared to behave as intermediates in comparison to the aforementioned examples. In addition to bacterial intrinsic mechanism, the regulation process of the hemolysin activity is usually associated with the synthesis of other virulence factors: for example, a common regulator for virulence factors is mediated by the same gene regulator which responds to environmental stimuli (including hemolysins) (Jonsson and Wadstrom, 1993; Regassa et al., 1992). Bacterial expression of hemolysin genes was also related to respond to changes in oxygen levels, the redox potential, and glutathione concentration of the environment (Bannan et al., 1993; Karunakaran and Holt, 1993; Williams and Austin, 1992). However, intrinsic events of the bacterial regulation and its environmental stimuli could also be elucidated by hematological and biochemical characterization (for example, human and animal blood agar) and bacterial gene expression studies (for example, hemolysins and virulence factors).

The evaluation of *in vitro* hemolysis activity within each subpopulation of oxacillin-resistant *S. aureus* (that is, *taxa* A, B and C) also pointed to CRBA culture medium as being favorable to the expression of hemolysins, as over 70% of bacterial isolates of each subpopulation

Table 8. Percent index of the morphological features of bacterial colonies within and among distantly genetically related populations (*taxa* A, B and C) of oxacillin-resistant *S. aureus* on thirteen different types of blood agar plates.

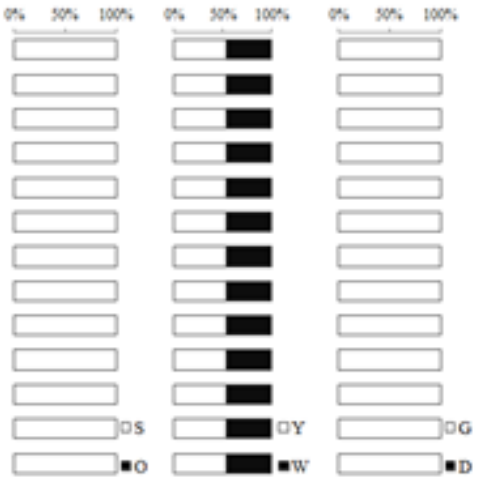
Culture media	Morphology of colonies						
	S	O	Y	W	G	D	
Taxon A (60 isolates /43 strains) – SO ^{A}, YW ^{A} and GD ^{A,B}							
DSBA commercial	98.3	1.7	60	40	98.3	1.7	
CSBA	98.3	1.7	60	40	98.3	1.7	
CRBA	98.3	1.7	60	40	98.3	1.7	
CHBA	98.3	1.7	60	40	98.3	1.7	
CBBA	98.3	1.7	60	40	98.3	1.7	
CHuBA A^{+}	98.3	1.7	60	40	98.3	1.7	
CHuBA A^{-}	98.3	1.7	60	40	98.3	1.7	
CHuBA B^{+}	98.3	1.7	60	40	98.3	1.7	
CHuBA B^{-}	98.3	1.7	60	40	98.3	1.7	
CHuBA AB^{+}	98.3	1.7	60	40	98.3	1.7	
CHuBA AB^{-}	98.3	1.7	60	40	98.3	1.7	
CHuBA O^{+}	98.3	1.7	60	40	98.3	1.7	
CHuBA O^{-}	98.3	1.7	60	40	98.3	1.7	
Taxon B (33 isolates /30 strains) – SO ^{B}, YW ^{B} and GD ^{A}							
DSBA commercial	100	-	42.4	57.6	97	3	
CSBA	100	-	42.4	57.6	97	3	
CRBA	100	-	42.4	57.6	97	3	
CHBA	100	-	42.4	57.6	97	3	
CBBA	100	-	42.4	57.6	97	3	
CHuBA A^{+}	100	-	42.4	57.6	97	3	
CHuBA A^{-}	100	-	42.4	57.6	97	3	
CHuBA B^{+}	100	-	42.4	57.6	97	3	
CHuBA B^{-}	100	-	42.4	57.6	97	3	
CHuBA AB^{+}	100	-	42.4	57.6	97	3	
CHuBA AB^{-}	100	-	42.4	57.6	97	3	
CHuBA O^{+}	100	-	42.4	57.6	97	3	
CHuBA O^{-}	100	-	42.4	57.6	97	3	
Taxon C (7 isolates /6 strains) – SO ^{B}, YW ^{B} and GD ^{B}							
DSBA commercial	100	-	53.8	46.2	100	-	
CSBA	100	-	53.8	46.2	100	-	
CRBA	100	-	53.8	46.2	100	-	
CHBA	100	-	53.8	46.2	100	-	
CBBA	100	-	53.8	46.2	100	-	
CHuBA A^{+}	100	-	53.8	46.2	100	-	
CHuBA A^{-}	100	-	53.8	46.2	100	-	
CHuBA B^{+}	100	-	53.8	46.2	100	-	
CHuBA B^{-}	100	-	53.8	46.2	100	-	
CHuBA AB^{+}	100	-	53.8	46.2	100	-	
CHuBA AB^{-}	100	-	53.8	46.2	100	-	
CHuBA O^{+}	100	-	53.8	46.2	100	-	
CHuBA O^{-}	100	-	53.8	46.2	100	-	

The letters S, O, Y, W, G and D correspond to shiny, opaque, yellow, white, glossy and dry, respectively. The three graphics to the right correspond to the data of each *taxon* from Table 8. The letters ^{A} and ^{B} correspond to the Tukey grouping.

were able to produce hemolysins. In general, the CSBA, CHBA, CBBA and commercial DSBA culture media displayed frequencies of hemolysins expression much lower than those observed for CRBA, and most

Table 9. Percent index of the morphological features of bacterial colonies within and among *clusters* moderately related and/or distantly genetically related (*clusters* of I to XV) of oxacillin-resistant *S. aureus* on thirteen different types of blood agar plates.

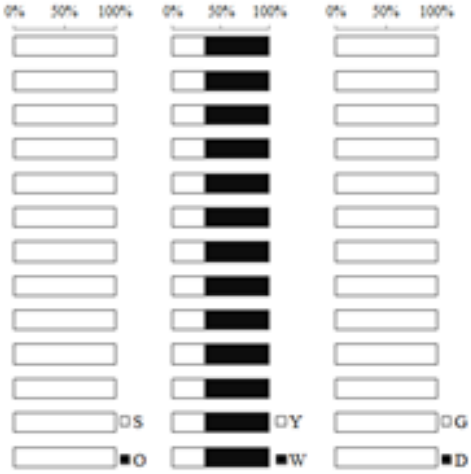
Culture media	Morphology of colonies S	O	Y	W	G	D
Cluster I (13 isolates /11 strains) – SO [A], YW [C] and GD [B]						
DSBA commercial	100	-	53.8	46.2	100	-
CSBA	100	-	53.8	46.2	100	-
CRBA	100	-	53.8	46.2	100	-
CHBA	100	-	53.8	46.2	100	-
CBBA	100	-	53.8	46.2	100	-
CHuBA A^+	100	-	53.8	46.2	100	-
CHuBA A^-	100	-	53.8	46.2	100	-
CHuBA B^+	100	-	53.8	46.2	100	-
CHuBA B^-	100	-	53.8	46.2	100	-
CHuBA AB^+	100	-	53.8	46.2	100	-
CHuBA AB^-	100	-	53.8	46.2	100	-
CHuBA O^+	100	-	53.8	46.2	100	-
CHuBA O^-	100	-	53.8	46.2	100	-
Cluster II (5 isolates /2 strains) – SO [A], YW [B] and GD [B]						
DSBA commercial	100	-	80	20	100	-
CSBA	100	-	80	20	100	-
CRBA	100	-	80	20	100	-
CHBA	100	-	80	20	100	-
CBBA	100	-	80	20	100	-
CHuBA A^+	100	-	80	20	100	-
CHuBA A^-	100	-	80	20	100	-
CHuBA B^+	100	-	80	20	100	-
CHuBA B^-	100	-	80	20	100	-
CHuBA AB^+	100	-	80	20	100	-
CHuBA AB^-	100	-	80	20	100	-
CHuBA O^+	100	-	80	20	100	-
CHuBA O^-	100	-	80	20	100	-
Cluster III (3 isolates /3 strains) – SO [A], YW [B, C] and GD [B]						
DSBA commercial	100	-	66.7	33.3	100	-
CSBA	100	-	66.7	33.3	100	-
CRBA	100	-	66.7	33.3	100	-
CHBA	100	-	66.7	33.3	100	-
CBBA	100	-	66.7	33.3	100	-
CHuBA A^+	100	-	66.7	33.3	100	-
CHuBA A^-	100	-	66.7	33.3	100	-
CHuBA B^+	100	-	66.7	33.3	100	-
CHuBA B^-	100	-	66.7	33.3	100	-
CHuBA AB^+	100	-	66.7	33.3	100	-
CHuBA AB^-	100	-	66.7	33.3	100	-
CHuBA O^+	100	-	66.7	33.3	100	-
CHuBA O^-	100	-	66.7	33.3	100	-



The letters S, O, Y, W, G and D correspond to shiny, opaque, yellow, white, glossy and dry, respectively. The three graphics to the right correspond to the data of each *cluster* from Table 9, respectively. The letters [A], [B], [C], [D], [E] and [F] correspond to the Tukey grouping.

Table 9, Contd.

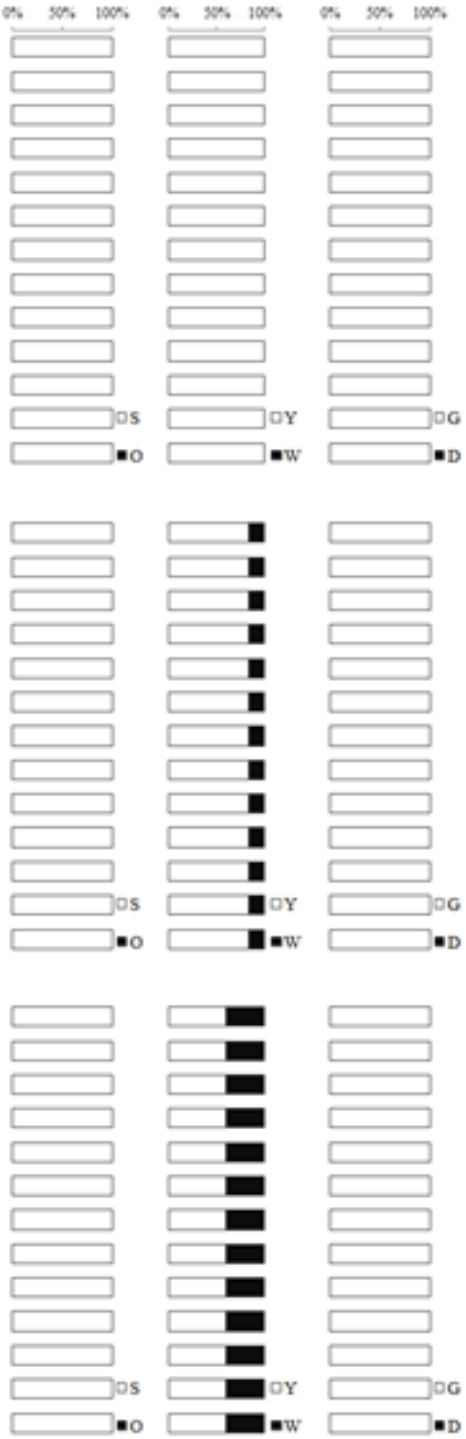
Culture media	Morphology of colonies S	O	Y	W	G	D
Cluster IV (3 isolates /3 strains) – SO [A], YW [D, E] and GD [B]						
DSBA commercial	100	-	33.3	66.7	100	-
CSBA	100	-	33.3	66.7	100	-
CRBA	100	-	33.3	66.7	100	-
CHBA	100	-	33.3	66.7	100	-
CBBA	100	-	33.3	66.7	100	-
CHuBA A^+	100	-	33.3	66.7	100	-
CHuBA A^-	100	-	33.3	66.7	100	-
CHuBA B^+	100	-	33.3	66.7	100	-
CHuBA B^-	100	-	33.3	66.7	100	-
CHuBA AB^+	100	-	33.3	66.7	100	-
CHuBA AB^-	100	-	33.3	66.7	100	-
CHuBA O^+	100	-	33.3	66.7	100	-
CHuBA O^-	100	-	33.3	66.7	100	-
Cluster V (4 isolates /3 strains) – SO [A], YW [F] and GD [B]						
DSBA commercial	100	-	-	100	100	-
CSBA	100	-	-	100	100	-
CRBA	100	-	-	100	100	-
CHBA	100	-	-	100	100	-
CBBA	100	-	-	100	100	-
CHuBA A^+	100	-	-	100	100	-
CHuBA A^-	100	-	-	100	100	-
CHuBA B^+	100	-	-	100	100	-
CHuBA B^-	100	-	-	100	100	-
CHuBA AB^+	100	-	-	100	100	-
CHuBA AB^-	100	-	-	100	100	-
CHuBA O^+	100	-	-	100	100	-
CHuBA O^-	100	-	-	100	100	-
Cluster VI (8 isolates /6 strains) – SO [A], YW [C] and GD [B]						
DSBA commercial	100	-	62.5	37.5	100	-
CSBA	100	-	62.5	37.5	100	-
CRBA	100	-	62.5	37.5	100	-
CHBA	100	-	62.5	37.5	100	-
CBBA	100	-	62.5	37.5	100	-
CHuBA A^+	100	-	62.5	37.5	100	-
CHuBA A^-	100	-	62.5	37.5	100	-
CHuBA B^+	100	-	62.5	37.5	100	-
CHuBA B^-	100	-	62.5	37.5	100	-
CHuBA AB^+	100	-	62.5	37.5	100	-
CHuBA AB^-	100	-	62.5	37.5	100	-
CHuBA O^+	100	-	62.5	37.5	100	-
CHuBA O^-	100	-	62.5	37.5	100	-



CHuBA culture media displayed a frequency between those of CRBA and other BA. As a result, such types of BA are less suited to routine use by the laboratories during the microbiological diagnosis of *S. aureus*. These

Table 9, Contd.

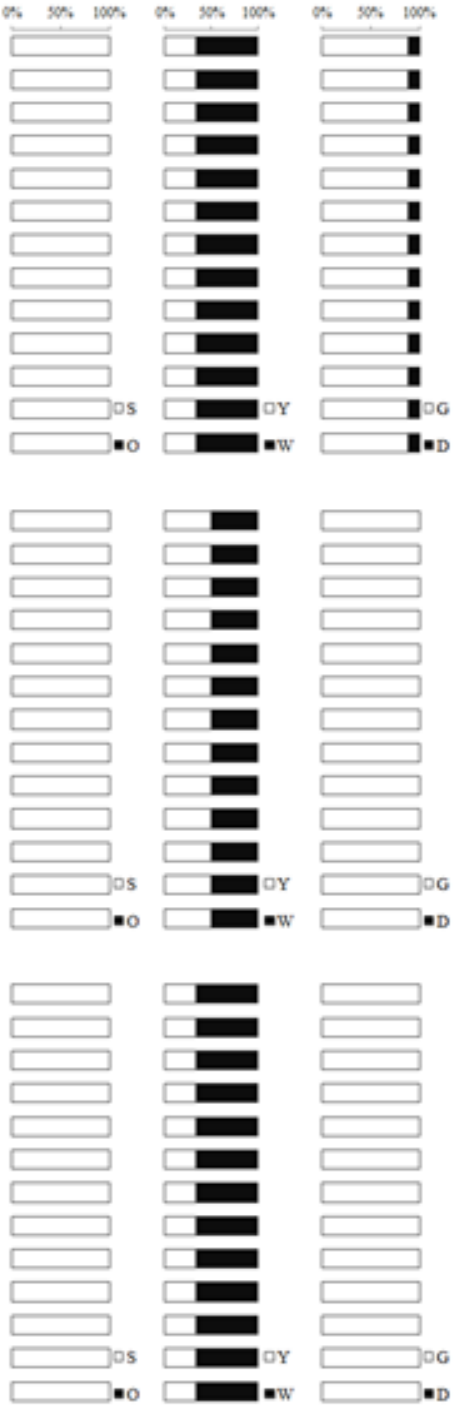
Culture media	Morphology of colonies S	O	Y	W	G	D
Cluster VII (3 isolates /3 strains) – SO [A], YW [A] and GD [B]						
DSBA commercial	100	-	100	-	100	-
CSBA	100	-	100	-	100	-
CRBA	100	-	100	-	100	-
CHBA	100	-	100	-	100	-
CBBA	100	-	100	-	100	-
CHuBA A^+	100	-	100	-	100	-
CHuBA A^-	100	-	100	-	100	-
CHuBA B^+	100	-	100	-	100	-
CHuBA B^-	100	-	100	-	100	-
CHuBA AB^+	100	-	100	-	100	-
CHuBA AB^-	100	-	100	-	100	-
CHuBA O^+	100	-	100	-	100	-
CHuBA O^-	100	-	100	-	100	-
Cluster VIII (12 isolates /3 strains) – SO [A], YW [A, B] and GD [B]						
DSBA commercial	100	-	83.3	16.7	100	-
CSBA	100	-	83.3	16.7	100	-
CRBA	100	-	83.3	16.7	100	-
CHBA	100	-	83.3	16.7	100	-
CBBA	100	-	83.3	16.7	100	-
CHuBA A^+	100	-	83.3	16.7	100	-
CHuBA A^-	100	-	83.3	16.7	100	-
CHuBA B^+	100	-	83.3	16.7	100	-
CHuBA B^-	100	-	83.3	16.7	100	-
CHuBA AB^+	100	-	83.3	16.7	100	-
CHuBA AB^-	100	-	83.3	16.7	100	-
CHuBA O^+	100	-	83.3	16.7	100	-
CHuBA O^-	100	-	83.3	16.7	100	-
Cluster IX (5 isolates /4 strains) – SO [A], YW [C] and GD [B]						
DSBA commercial	100	-	60	40	100	-
CSBA	100	-	60	40	100	-
CRBA	100	-	60	40	100	-
CHBA	100	-	60	40	100	-
CBBA	100	-	60	40	100	-
CHuBA A^+	100	-	60	40	100	-
CHuBA A^-	100	-	60	40	100	-
CHuBA B^+	100	-	60	40	100	-
CHuBA B^-	100	-	60	40	100	-
CHuBA AB^+	100	-	60	40	100	-
CHuBA AB^-	100	-	60	40	100	-
CHuBA O^+	100	-	60	40	100	-
CHuBA O^-	100	-	60	40	100	-



data suggest support the use of CRBA medium in the characterization and microbiological diagnosis of oxacillin-resistant *S. aureus*, especially during routine hemolytic activity detection, regardless of the (sub)

Table 9. Contd.

Culture media	Morphology of colonies S	O	Y	W	G	D
Cluster X (9 isolates /9 strains) – SO [A], YW [D, E] and GD [A]						
DSBA commercial	100	-	33.3	66.7	88.9	11.1
CSBA	100	-	33.3	66.7	88.9	11.1
CRBA	100	-	33.3	66.7	88.9	11.1
CHBA	100	-	33.3	66.7	88.9	11.1
CBBA	100	-	33.3	66.7	88.9	11.1
CHuBA A^+	100	-	33.3	66.7	88.9	11.1
CHuBA A^-	100	-	33.3	66.7	88.9	11.1
CHuBA B^+	100	-	33.3	66.7	88.9	11.1
CHuBA B^-	100	-	33.3	66.7	88.9	11.1
CHuBA AB^+	100	-	33.3	66.7	88.9	11.1
CHuBA AB^-	100	-	33.3	66.7	88.9	11.1
CHuBA O^+	100	-	33.3	66.7	88.9	11.1
CHuBA O^-	100	-	33.3	66.7	88.9	11.1
Cluster XI (2 isolates /2 strains) – SO [A], YW [C, D] and GD [B]						
DSBA commercial	100	-	50	50	100	-
CSBA	100	-	50	50	100	-
CRBA	100	-	50	50	100	-
CHBA	100	-	50	50	100	-
CBBA	100	-	50	50	100	-
CHuBA A^+	100	-	50	50	100	-
CHuBA A^-	100	-	50	50	100	-
CHuBA B^+	100	-	50	50	100	-
CHuBA B^-	100	-	50	50	100	-
CHuBA AB^+	100	-	50	50	100	-
CHuBA AB^-	100	-	50	50	100	-
CHuBA O^+	100	-	50	50	100	-
CHuBA O^-	100	-	50	50	100	-
Cluster XII (3 isolates /3 strains) – SO [A], YW [D, E] and GD [B]						
DSBA commercial	100	-	33.3	66.7	100	-
CSBA	100	-	33.3	66.7	100	-
CRBA	100	-	33.3	66.7	100	-
CHBA	100	-	33.3	66.7	100	-
CBBA	100	-	33.3	66.7	100	-
CHuBA A^+	100	-	33.3	66.7	100	-
CHuBA A^-	100	-	33.3	66.7	100	-
CHuBA B^+	100	-	33.3	66.7	100	-
CHuBA B^-	100	-	33.3	66.7	100	-
CHuBA AB^+	100	-	33.3	66.7	100	-
CHuBA AB^-	100	-	33.3	66.7	100	-
CHuBA O^+	100	-	33.3	66.7	100	-
CHuBA O^-	100	-	33.3	66.7	100	-



population genetic classifications.

In addition, during the *clusters* analysis of oxacillin-resistant *S. aureus*, for almost all *clusters,* the majority of the isolates expressed *in vitro* hemolysins on CRBA culture medium, although there was variation in the *Pz* and in terms of obvious and/or faint character, and yet,

Table 9, Contd.

Culture media	Morphology of colonies S	O	Y	W	G	D
Cluster XIII (4 isolates /2 strains) – SO [A], YW [E] and GD [B]						
DSBA commercial	100	-	25	75	100	-
CSBA	100	-	25	75	100	-
CRBA	100	-	25	75	100	-
CHBA	100	-	25	75	100	-
CBBA	100	-	25	75	100	-
CHuBA A^+	100	-	25	75	100	-
CHuBA A^-	100	-	25	75	100	-
CHuBA B^+	100	-	25	75	100	-
CHuBA B^-	100	-	25	75	100	-
CHuBA AB^+	100	-	25	75	100	-
CHuBA AB^-	100	-	25	75	100	-
CHuBA O^+	100	-	25	75	100	-
CHuBA O^-	100	-	25	75	100	-
Cluster XIV (2 isolates /2 strains) – SO [A], YW [C, D] and GD [B]						
DSBA commercial	100	-	50	50	100	-
CSBA	100	-	50	50	100	-
CRBA	100	-	50	50	100	-
CHBA	100	-	50	50	100	-
CBBA	100	-	50	50	100	-
CHuBA A^+	100	-	50	50	100	-
CHuBA A^-	100	-	50	50	100	-
CHuBA B^+	100	-	50	50	100	-
CHuBA B^-	100	-	50	50	100	-
CHuBA AB^+	100	-	50	50	100	-
CHuBA AB^-	100	-	50	50	100	-
CHuBA O^+	100	-	50	50	100	-
CHuBA O^-	100	-	50	50	100	-
Cluster XV (4 isolates /3 strains) – SO [A], YW [C, D] and GD [B]						
DSBA commercial	100	-	50	50	100	-
CSBA	100	-	50	50	100	-
CRBA	100	-	50	50	100	-
CHBA	100	-	50	50	100	-
CBBA	100	-	50	50	100	-
CHuBA A^+	100	-	50	50	100	-
CHuBA A^-	100	-	50	50	100	-
CHuBA B^+	100	-	50	50	100	-
CHuBA B^-	100	-	50	50	100	-
CHuBA AB^+	100	-	50	50	100	-
CHuBA AB^-	100	-	50	50	100	-
CHuBA O^+	100	-	50	50	100	-
CHuBA O^-	100	-	50	50	100	-

regardless of the number of isolates or strains present in the *clusters* (average of 5.33 ± 3.51 isolates by *cluster*; average of 3.93 ± 2.68 strains by cluster). These data reinforce the hypothesis above about using CRBA medium in the characterization and microbiological diagnosis of *S. aureus*, regardless of the *clusters* are genetically moderately or distantly related and possibly not related epidemiologically. This information also indicates the existence of two or more genetically identical (same strains [ETs]) or highly related (common

ancestor) isolates that are possibly related from an epidemiological point of view and with the potential for phenotypic expression of virulence, especially *in vitro* hemolysins, simultaneously due to their intrinsic molecular metabolisms and under the influence of the external environment. The determination of such environmental influence can be based on the observations of variability of hemolytic expression on human and animal BA media by a single isolate or strain.

A comparative study between CSBA (citrated sheep blood agar), CHuBA (citrated human blood agar), DHBA (defibrinated horse blood agar) and DSBA (defibrinated sheep blood agar) used for the isolation and antimicrobial susceptibility testing of the strains of *S. pneumoniae, S. pyogenes* and *S. aureus* revealed similar colony count values on all culture media, and size of the colonies was generally smaller and accompanied by an absence or a deficit in hemolysin expression on CHuBA for all three species of microorganisms (Russell et al., 2006). At least for *S. aureus*, our size results support these findings, as the CHuBA produces a smaller colony diameter than CSBA, CHBA and CBBA. However, in contrast to the hemolysis findings, a large number of isolates and/or clinical strains of oxacillin-resistant *S. aureus* were potentially capable of producing hemolysis *in vitro* on CRBA plates, followed by CHuBA, CSBA, CHBA, CBBA and commercial DSBA whose hemolysis were quantitatively reduced on these last types of animal BA medium. Given that the size of the colony, the colony morphology, and hemolysis are essentially critical for identification of *S. pneumoniae, S. pyogenes* and *S. aureus*, Russell et al. (2006) discussed the large possibility that these microorganisms can be neglected or mistakenly identified when grown on CHuBA, especially when other microorganisms are present in biological samples, such as those from the upper respiratory tract or skin. In addition, the CHuBA demonstrated a performance in antibiotic susceptibility tests that was insufficient when compared with SBA (sheep blood agar). These findings have profound implications in developing countries where expired human blood is commonly used as a culture media supplement. Accordingly, it is likely that clinical laboratory diagnostics of infectious diseases are underestimated by laboratories using CHuBA culture medium (Russell et al., 2006). Therefore, Hu MHA (human Mueller-Hinton blood agar) plates should not be recommended for antimicrobial susceptibility tests or isolation of *S. pneumoniae, S. pyogenes* and *S. aureus* (Anand et al*.*, 2000; Centers for Disease Control and Prevention, 1998; Egwuatu et al., 2014; Gratten et al., 1994; Johnson et al., 1996; Satzke et al., 2010), despite their routine use by developing countries.

Another study published on the isolation of *Bordetella pertussis* on different BA culture media compared Petri dishes containing HBA (horse blood agar), DSBA (defibrinated sheep blood agar) and anticoagulated HuBA (human blood agar) (Hoppe and Schlagenhauf, 1989).

This comparison demonstrated that the HuBA was inferior to the HBA and DSBA. Despite the lack of clarification on the findings related to HuBA, studies in the literature have suggested that human blood can contain antibiotics, antibodies or other anti-infective agents (Johnson et al., 1996), and the lack of hemolysis on HuBA may also be due to age of red blood cells in human blood that has expired or other factors. It is important to note the similar microbiological findings using the DSBA and CSBA dishes (Russell et al., 2006), although it is reported in the literature that citrate displays antibacterial characteristics (Young and Foegeding, 1993; Phillips, 1999). These findings strengthen the hypothesis that CSBA can be safely used for the isolation of *S. pneumoniae, S. pyogenes* and *S. aureus*, at a proportion of 1:10 citrate:blood, although it remains unknown whether smaller proportions may affect the patterns of growth and susceptibility of these microorganisms. Accordingly, care should be taken during collection to ensure the correct proportion of blood, and additional studies should examine this issue (Russell et al., 2006). Other studies have demonstrated that defibrinated pig blood and goat blood are viable alternatives as a supplement for *S. pneumoniae* culture media (Young and Foegeding, 1993; Phillips, 1999). These findings support the increased possibility of the acceptability of citrated blood from animals other than sheep (Russell et al., 2006).

The effect of the different blood (that is, goat, sheep, cow, chicken, rabbit and fresh human blood) on the cultural and morphological characteristics of the bacterial isolates (*P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and β-heamolytic and non-haemolytic *Streptococcus*) was recently determined (Egwuatu et al., 2014). All these blood agars supported the growth of all these bacterial isolates and without significant difference in the morphology and cultural characteristics (that is, size, colour, pigmentation, elevation, consistency and shape of the colonies). However, some isolates (especially for *S. aureus*) showed some differences in their abilities to distinguish α- and β-haemolytic patterns dependent on blood agar types (Egwuatu et al., 2014).

The diagnostic morphological aspects of the colonies (that are, shiny or opaque, yellow or white, glossy or dry) were invariably displayed in the total population, in subpopulations (*taxa*) and in the *clusters* of isolates of oxacillin-resistant *S. aureus*, regardless of the BA culture media human and animal. Shiny and glossy bacterial colonies predominated in the total population (Table 7) in the (sub) populations (*taxa*) (Table 8) and in some isolates cluster (Table 9), and the bacterial colony colors of yellow or white were often similar. Although each taxonomic rank of isolates ORSA (*taxa* and *clusters*) displayed these morphological aspects regardless of the type of BA media, significant differences were observed between (*i*) *taxa* B and A or C and A regarding the shiny/opaque aspects and yellow/white coloration, (*ii*) the

taxa B and C regarding the glossy/dry aspect, (*iii*) the *clusters* in nine distinct situations regarding the yellow/white coloration and (*iv*) the *cluster* X compared to the other *clusters* regarding the glossy/dry aspect. No difference was observed between the *clusters* regarding the shiny/opaque aspect. These results indicate that human and animal BA culture media does not influence the morphological aspects of the colonies in terms of appearance, particularly where the colonies are shiny, glossy and either yellow or white. These aspects may be observed independently (*i*) in (sub)populations regardless of whether they are related and genetically and epidemiologically distant, and (*ii*) they may be observed in *clusters* that are moderately related or distantly genetically, and even possibly unrelated epidemiologically. However, certain *clusters* could harbor isolates/strains that are predominantly yellow or white without any exclusivity for this phenotype. These findings indicate the existence of two or more genetically identical (same strain ^{ET}) or highly related (common ancestor) isolates that are possibly related from an epidemiological point of view that may share the same wild species-specific phenotypes related to appearance (that is, especially shiny, glossy and yellow or white), without any influence from the external environment. Such a statement may be based on the observations of phenotypic invariance in the appearance of colonies on human and animal BA culture media for the same isolate or strain.

These characteristically invariant morphological aspects were also demonstrated by Russell and associates (2006), which examined only two strains of *S. aureus* [that is, *S. aureus* ATCC 25923: opaque-white-glossy (HBA), opaque-white-glossy (CSBA), opaque-white-glossy (DSBA) and opaque-white-glossy (HuBA); *S. aureus* ATCC 29213: Opaque-yellow-glossy (HBA), opaque-yellow-glossy (CSBA), opaque-yellow-glossy (DSBA) and opaque-yellow-glossy (HuBA)] on different types of BA media. However, this invariability cannot be confirmed for *S. pneumoniae* and *S. pyogenes* as currently reported [that is, *S. pneumoniae* ATCC 6305: shiny-grey (HBA), mucoid-grey (CSBA), dull-grey (DSBA) and dull-grey (HuBA); *S. pneumoniae* ATCC 49619: Shiny-mucoid-grey (HBA), dry-grey (CSBA), dry-grey (DSBA) and shiny-grey (HuBA); *S. pyogenes* ATCC 19615: Glossy-white (HBA), dry-grey-white (CSBA), dry-grey (DSBA) and glossy-white (HuBA); *S. pyogenes* strain JC20: glossy-white (HBA), glossy-white (CSBA), glossy-white (DSBA), and glossy-white (HuBA)] (Russell et al., 2006).

The present study evaluates the performance characteristics of bacterial growth (that is, the size of the ∅ and the appearance of colonies) and the production of *in vitro* hemolysis of a partial population of oxacillin-resistant *S. aureus* isolates (that is, dental origin from a molecular epidemiological study in progress), grown on non-commercially sourced human and animal citrated BA culture media [that is, citrated sheep BA (CSBA), citrated bovine BA (CBBA), citrated horse BA (CHBA), citrated rabbit BA (CRBA), citrated human BA O^- (CHuBA O^-), citrated human BA O^+ (CHuBA O^+), citrated human BA A^- (CHuBA A^-), citrated human BA A^+ (CHuBA A^+), citrated human BA B^- (CHuBA B^-), citrated human BA B^+ (CHuBA B^+), citrated human BA AB^- (CHuBA AB^-) and citrated human BA AB^+ (CHuBA AB^+)] and commercially available defibrinated sheep agar (commercial DSBA). The identification of genotypes and genetic relationship between strains, *clusters* and *taxa*, were determined using the MLEE method, clustering and genetic analyses to establish a possible correlation between the phenotypic and genotypic characteristics. The MLEE method has proved to be a powerful tool for the typing of *S. aureus* in epidemiological studies and possess a high discriminatory power and reproducibility. However, given our particular research goals, no epidemiologic inference was performed in this study.

In the total bacterial population, phenotypic variability was observed between different strains and even between different isolates belonging to the same strain depending on the BA media used (that is, variability in appearance, in the size of the colony and in the β-hemolytic activity). The diameter of the colonies was observed to have variations: (i) in the total population of isolates and (ii) within and between taxonomic *ranks* (that is, *taxa* or *clusters*) depending on the BA media used. As for colony appearance (that is, shiny/opaque, yellow/white and glossy/dry), the BA media did not appear to influence colonial morphology among isolates/strains or taxonomic *ranks* (that is, *taxa* or *clusters*). However, certain ranks did harbor strains that were primarily yellow or white without any exclusivity for this phenotype. In regards to hemolytic activity, the rabbit BA favored the expression of hemolysins, followed by the human BA media and the BA media from other animals. The expression of hemolysis revealed intrinsic characteristics in each taxonomic *rank* and differences between them (that is, *taxa* or *clusters*), with the hemolysis occurrence being dependent the BA media used. These data suggest that the hemolysin expression by *S. aureus* may be favored through the use of a particular type of BA culture to the detriment of colonial growth potential, particularly the CRBA culture media and *vice-versa* (that is, expression partially favored or blocked depending on the used BA medium but with a greater associated potential for colonial growth, especially for CHBA and CBBA culture media). This study also suggests the use of the CRBA media in the characterization and microbiological diagnosis of oxacillin-resistant *S. aureus*, especially during routine detection of hemolytic activity and large-scale studies, regardless of the taxonomic classifications of the isolates (that is, *taxa* and/or cluster). In addition, phenotypic and genotypic correlation studies of bacterial population groups (that is, the groups of microbial genera of medical importance that

require a blood source) and the use of the BA culture media could elucidate (i) the microbial behavior *in vitro* and (ii) facilitate the standardization of methodology, whether in terms of isolation or in terms of species-specific phenotypic characterization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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