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ORIGINAL ARTICLE

Genetic relationships and virulence factors in *Staphylococcus aureus* isolated from raw poultry in South Brazil

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Abstract The genetic relationships in *Staphylococcus aureus* isolated from raw poultry obtained from various Brazilian broiler chicken processors were analyzed using repetitive extragenic palindromic-polymerase chain reaction (rep-PCR). The distribution of accessory gene regulator (*agr*) groups was determined, and the presence of biofilm-associated genes and phenotypic features, including biofilm formation and proteolytic, lipolytic, and β-hemolytic activities, was assessed. Isolates were grouped into three major clusters based on rep-PCR fingerprints typed with RW3A primer. The *agr* group I was the most common genotype identified (86.21 %), followed by groups II (10.34 %) and III (3.45 %). All strains were positive for the *sasG* gene; the next most frequent genes were *icaA* (93.1 %) and *atlA* (51.72 %). Twenty-six of the 29 isolates were biofilm producers. In this study, 96.55 %, 72.41 %, and 62.06 % of the isolates displayed lipolytic, β-hemolytic, and proteolytic activity, respectively. In conclusion, the rep-PCR results suggested a clonal relationship among the *S. aureus* isolated from raw poultry produced by different broiler chicken processors. Our results also showed that most isolates belonged to *agr* group I. The presence of biofilm-forming *S. aureus* strains in raw poultry, their ability to harbor biofilm-associated genes, and the spoilage features that they exhibit are indicative of their pathogenic potential, and may represent a serious problem in the food processing industry.

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Introduction

Staphylococcus aureus are Gram-positive cocci that inhabit the skin and mucous membranes of humans and animals (Hanselman et al. 2009; Gharsa et al. 2012; Gundogan et al. 2012; Gutiérrez et al. 2012). This species causes diseases through two different mechanisms: multiplying and spreading widely in tissues or production of extracellular enzymes and toxins. Staphylococcal food poisoning, a form of gastroenteritis characterized by rapid onset of symptoms, typically occurs after ingestion of food—usually meat, processed meat, milk, or dairy products—contaminated with a toxin produced by *S. aureus* (Pereira et al. 2009; Pu et al. 2011; Moura et al. 2012; Martins et al. 2013; Njage et al. 2013). The presence of *S. aureus* in food is often attributed to improper handling during processing and packaging of food, or to infection or colonization of animals that can contaminate carcasses during the slaughter process (Devita et al. 2007; Greig and Ravel 2009; Pu et al. 2011; Weese et al. 2010).

Members of the *Staphylococcus* genus express a wide range of virulence factors, including cell wall components, biofilm formation proteins, proteases, lipases, coagulase, hemolysins, nuclease, fibrinolysin, enterotoxins, and toxic shock syndrome toxin (Novick et al. 2001; Peacock et al. 2002; Gundogan et al. 2012; Moura et al. 2012; Tang et al. 2013). Biofilms are a serious problem in many sectors of the food industry and are known to play a role in chronic infections (Jessen and Lammert 2003; Otto 2013; Giaouris et al. 2014). In food processing, biofilms are a potential source of product contamination that may lead to food spoilage and can cause issues through serious fouling of equipment. Biofilm formation in *S. aureus* is a complex process involving multiple

phases. The first phase requires surface adhesin and autolysin proteins in the bacterial membrane, one of which—the autolysin A (AtlA)—is encoded by the *atlA* gene. The maturation phase is characterized by the production of extracellular factors that mediate contact with the bacterial cell. *Staphylococcus* spp. can mediate cell–cell adhesion using the polysaccharide intercellular adhesin (PIA) encoded by the operon *icaADBC* (PIA-dependent mechanism) or other proteins (PIA-independent mechanisms), such as the *S. aureus* surface protein G (SasG) (Otto 2013).

The pattern of virulence genes and genetic polymorphisms in bacteria can be used to determine the origin of the isolates or their pathogenicity. The genetic relationship between microorganisms can be determined by repetitive element polymorphism-polymerase chain reaction (rep-PCR), which is a reproducible method that has been used for outbreak investigations and discrimination of *S. aureus* strains (Reinoso et al. 2008; Sadoyama et al. 2008; Njage et al. 2013). The genetic polymorphism of the accessory gene regulator (*agr*) system of *S. aureus* is responsible for controlling the expression of many genes that code for virulence factors. The *agr* locus comprises the *agrA*, *agrB*, *agrC*, and *agrD* genes, which are co-transcribed (RNAII), and can be used to categorize *S. aureus* strains into four groups (I, II, III, and IV). These groups have been associated with patterns of disease and antibiotic resistance in clinical isolates in several studies (Jarraud et al. 2002; Sakoulas et al. 2003; Moise-Broder et al. 2004). These allelic groups have also been evaluated in strains isolated from clinical and subclinical mastitis in cattle and sheep, as well as in food products (Spanu et al. 2012; Almeida et al. 2013; Bibalan et al. 2014). To date, there have been no reports on the relationship between genetic polymorphisms of the *agr* locus or genetic polymorphisms of *S. aureus* isolated from raw poultry in the South Region of Brazil.

The aim of this study was to use rep-PCR to explore the genetic relationships between *Staphylococcus aureus* strains isolated from raw poultry produced by different Brazilian broiler chicken processors in South Brazil. We also evaluated the distribution of the accessory gene regulator (*agr*) and assessed biofilm-associated genes and spoilage and phenotypic features, including proteolytic, lipolytic, and β-hemolytic activities.

Materials and methods

Bacterial strains and molecular identification of species by PCR

Strains of *S. aureus* under investigation (n=29) were isolated from chilled and frozen raw poultry parts produced by seven different Brazilian broiler chicken processors, and were identified using conventional biochemical tests. The poultry samples were collected in April and May 2011 in South Brazil (Martins et al. 2013). Genomic DNA was extracted from isolates following the protocol described by Moura et al. (2012). All strains were confirmed as *S. aureus* by polymerase chain reaction (PCR), using a species-specific primer based on the *nuc* gene. Primer sequences are listed in Table 1. The isolates were stored in a 10 % (w/v) solution of skim milk (Difco™; Becton, Dickinson and Co., Sparks, MD, USA) and 10 % (v/v) glycerol (Neon Comercial Ltd, São Paulo, Brazil), and frozen at −20 °C.

PCR-restriction fragment length polymorphism analysis (PCR-RFLP) of agr groups

PCR-RFLP was used to identify *agr* groups. The primer sequences were designed to amplify a 1,899-base-pair (bp) fragment containing a conserved DNA sequence of *agr* groups I, II, III, and IV (Table 1). Sequences of the four *agr* groups of *S. aureus* were retrieved from the GenBank sequence database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) [GenBank accession nos. X52543 (*agr* group I), AF001782 (*agr* group II), AF001783 (*agr* group III), and AF288215 (*agr* group IV)]. Amplification

Table 1 Primers used in the PCRs carried out in this work

Primer	Sequence (5′–3′)	Annealing temperature	Amplicon (bp)	Reference
nuc-F	GCGATTGATGGTGATACGGT	60 °C	270 bp	This study
nuc-R	CAAGCCTTGACGAACTAAAGC			
agr-F	AACTTAGATCATATTCAATTTTTGC	60 °C	1889 bp	This study
agr-R	AGACATTGTCTGCATTATCAGC			
icaA-F	AAACTTGGTGCGGTTACAGG	54 °C	188 bp	Reiter et al. 2012
icaA-R	GTAGCCAACGTCGACAACTG			
atlA-F	CAGTTAGCAAGATTGCTCAAG	54 °C	1035 bp	Reiter et al. 2012
atlA-R	CCGTTACCTGTTTCTAATAGG			
sasG-F	ACCACAGGGTGTAGAAGCTAAATC	54 °C	188 bp	Reiter et al. 2012
sasG-R	CGAGC TTTTCTAA CCTTAGGTGTC			

bp, base pairs

was performed in a 25 μL mixture consisting of 150 ng of genomic DNA, 1.5 mM of $MgCl_2$, 1 μM of each primer, 200 μM of each dNTP, 1 U *Taq* DNA polymerase, and 1X reaction buffer. Amplification was carried out in an Eppendorf Mastercycler® personal 5332 thermocycler (Eppendorf AG, Hamburg, Germany), according to the following program: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product of 1,889 bp was submitted to digestion with the restriction endonuclease *Sca*I (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The DNA fragments obtained were resolved using electrophoresis on 1.5 % (w/v) agarose gel, visualized on a UV transilluminator, and photographed.

The polymorphisms of the *agr* operon were confirmed by DNA sequencing. Two randomly selected PCR products from each *agr* group were purified with Illustra™ GFX™ PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). Sequencing was carried out with the ABI PRISM® BigDye® Primer Cycle Sequencing Ready Reaction Kit in an ABI PRISM® 3100 genetic analyzer (Applied Biosystems®; Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The sequences obtained were compared to homologous nucleotide sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and aligned to these sequences (http://www.ncbi.nlm.nih.gov): X52543, AF001782, AF001783, and AF288215.

Molecular analysis by repetitive element polymorphism PCR (rep-PCR)

Fingerprints were generated by rep-PCR using a modification of the procedure described by Santos et al. (2001). PCR was performed in a 25 μl mixture consisting of 32.5 pmol of RW3A (5′-TCGCTAAAACAACGACACC-3′) primer (Del Vecchio et al. 1995), 250 μM of each dNTP (ABgene, Thermo Scientific), 1.25 U of *Taq* DNA polymerase (Invitrogen; Thermo Fisher), 10 mM of Tris–HCl (pH 9.2), 25 mM of KCl, 3 mM of $MgCl_2$ (Invitrogen), and 100 ng template DNA. Amplification was carried out in a thermocycler (AmpliTherm; Epicentre, Madison, WI, USA) according to the following program: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 94 °C for 1 min, annealing at 54 °C for 1.5 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

DNA fragments were separated on 2 % (w/v) agarose gels by electrophoresis, stained with ethidium bromide (0.5 μg/ml), visualized on a UV transilluminator, and photographed. After standardization with a molecular weight marker of 100 bp (Invitrogen), computer-aided gel analysis was carried out using the Gel-Pro Analyzer 32-bit software application (Media Cybernetics, Warrendale, PA, USA). DNA fingerprint patterns were analyzed using BioNumerics software (Applied Maths, Inc., Austin, TX, USA). Pearson correlation coefficients were calculated for the distance matrix, and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA).

Virulence factors

In vitro biofilm formation on polystyrene microplates

The microtiter-plate assay for biofilm formation was based on the method described by Stepanović et al. (2007). Briefly, bacteria were grown in tryptic soy broth (TSB; Oxoid Ltd., Basingstoke, UK) at 35 °C for 18 h. Polystyrene microplates were filled with 180 μl of TSB supplemented with 1 % glucose (TSBG) and 20 μl of a 10^8 CFU/ml culture, and incubated for 18 h at 35 °C. The optical density of the suspension was determined at 492 nm (OD_{492}) using a Biochrom Anthos 2010 microplate reader (Biochrom Ltd., Cambridge, UK). The optical density (OD) of each strain was determined by comparing the arithmetic mean of the absorbance of the wells with the mean absorbance of negative controls (OD_{nc}). The strains were categorized on the basis of their OD, as follows: non-biofilm producers ($OD_s \leq OD_{nc}$), weak biofilm producers ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm producers ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$), and strong biofilm producers ($4.OD_{nc} < OD_s$). All biofilm assays were run in eight replicates and repeated three times. Un-inoculated TSBG wells were used as negative controls and Gram-positive *S. epidermidis* ATCC 35984 as positive controls.

Detection of biofilm-forming genes by PCR

The presence of *icaA*, *sasG*, and *atlA* genes, which are involved in biofilm formation, was detected using the method described by Reiter et al. (2012). The primer sequences are listed in Table 1. The reactions were carried out in an Eppendorf Mastercycler® personal 5332 thermocycler (Eppendorf AG). The PCR products were analyzed using electrophoresis gel with 1.5 % agarose, stained with ethidium bromide solution, and viewed under ultraviolet light.

Hemolytic activity

Hemolytic activity was assessed by observation of the hemolysis zone around colonies after incubation for 24 h at 35 °C (Ruaro et al. 2013) on blood agar plates prepared with Mueller-Hinton agar (Biolife Italiana S.r.l., Milan, Italy) containing defibrinated sheep blood (final blood concentration: 5 % v/v).

Protease activity

Proteolytic activity was assessed using the method described by Ruaro et al. (2013), in which isolates were streaked onto skim milk agar (Oxoid Ltd., Basingstoke, UK) and incubated at 35 °C for 48 h. Protease production was indicated by the presence of transparent zones around the colony caused by degradation of milk caseins by the isolate being tested.

Lipase activity

Lipolytic activity was determined using a tributyrin agar base containing 1 % tributyrin (TBA; Oxoid Ltd.). The TBA plates were inoculated with isolates and incubated at 35 °C for 48 h. The presence of clear zones after incubation was indicative of lipase activity (Ruaro et al. 2013).

Results and discussion

Prevalence of agr groups in *Staphylococcus aureus* isolated from raw poultry

We detected three different *agr* groups in *S. aureus* isolated from raw poultry (Fig. 1). The most common genotype in our sample was the *agr* group I (86.21 %), followed by *agr* groups II (10.34 %) and III (3.45 %). No isolates carried *agr* group IV (Table 2). The polymorphism of the *agr* groups was confirmed by selecting six strains (two from each *agr* group) and sequencing them. After sequencing, these strains showed similarity to the reference groups as follows: *agr* group I, GenBank: X52543; *agr* group II, GenBank: AF001782; and *agr* group III, GenBank: AF001783.

The distribution of *agr* groups in *S. aureus* observed in this study was consistent with previous reports on *S. aureus* isolated from humans and other animal species (Gilot and Van Leeuwen 2004; Goerke et al. 2005; Gharsa et al. 2012; Almeida et al. 2013; Khan et al. 2014; Bibalan et al. 2014). The main *agr* group in *S. aureus* isolated from healthcare workers and food products was *agr* group I (Bibalan et al. 2014). Gharsa et al. (2012) showed that *agr* group I predominated (74 %) in *S. aureus* isolated from healthy donkeys. In addition, *agr* group I was also the most prevalent *agr* group in clinical strains (Gilot and Van Leeuwen 2004; Khan et al. 2014). The *agr* groups II and III were both present at low frequencies in our isolated strains. Similarly, Gharsa et al. (2012) observed low frequencies of *agr* II and III in *S. aureus* isolated from nasal swabs taken from healthy donkeys. The *agr* group II has been associated with hospital-acquired infections and decreased susceptibility to glycopeptides (Sakoulas et al. 2003; Moise-Broder et al. 2004). The a*gr* group III is involved in diseases such as toxic shock syndrome, necrotizing pneumonia, and infections caused by community-acquired methicillin-resistant *S. aureus* (CA-MRSA) (Gillet et al. 2002; Peacock et al. 2002). Isolates carrying *agr* group IV are rare. Some *agr* group IV strains are associated with exfoliative toxins, but in other cases it is not possible to detect the exfoliative toxin gene (Jarraud et al. 2002).

Molecular genotyping of *Staphylococcus aureus* isolated from raw poultry

rep-PCR amplification followed by dendrogram analysis was used to categorize isolates into three groups, designated A, B, and C (Fig. 2). Group A comprised 25 isolates with 100 % identity and one isolate with 92.0 % identity. Twenty-one of the 26 strains in this group were isolated from samples of the same brand (sample 2), purchased at different times. This distribution may indicate a clonal relationship among the *S. aureus* isolated from raw poultry produced by different Brazilian broiler chicken processors, which raises the possibility that these strains could have a single origin. The single strain isolated from sample 1 and two strains isolated from sample 4 had DNA band patterns that were different from

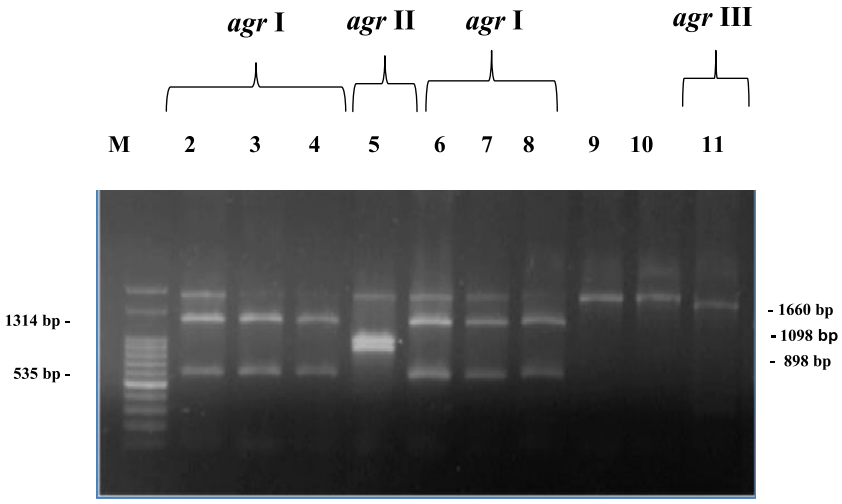

Fig. 1 Digestion of the PCR product for *agr* groups of *S. aureus* with the restriction enzyme *Sca*I. M: Molecular weight marker 100 bp ladder (Invitrogen). Lines 2, 3, 4, 6, 7, 8: *agr* Group I; Line 5 *agr* Group II; Lines 9 and 10: PCR product non-digested; Line 11: *agr* Group III

Table 2 Further discrimination of the amplified PCR product for the *agr* groups of *Staphylococcus aureus* by digestion with the restriction enzyme *Sca*I

agr group	RFLP product sizes (bp)	Isolates	
		n	%
I	1314–585	25	86.21
II	1001–898	3	10.34
IIII	1660–239	1	3.45
IV	1889	0	0

bp, base pairs

those of Group A. These results are in agreement with findings reported by Reinoso et al. (2008) and Njage et al. (2013), who also found low genetic variability in *S. aureus* strains isolated from food by rep-PCR.

The relationship between rep-PCR and *agr* groups is shown in Fig. 2. Group A consisted of all isolates from *agr* group I and one from *agr* group II (F4-2); group B comprised the single isolate in *agr* group III (F1-1), and group C consisted of the two *agr* group II (F4-1 and F4-3) isolates. A comparison of the results obtained using the two methods revealed a strong correlation between rep-PCR and *agr* group genotyping. Jarraud et al. (2002) demonstrated that, in terms of *agr* locus and RFLP genetic analysis, strains from *agr* groups III and IV were clearly individualized, whereas strains from *agr* groups I and II were partly superimposed.

Biofilm formation and frequency of related genes assessed by PCR

Of the 29 *S. aureus* isolates, 19 (65.52 %) were strong biofilm producers, 8 (27.58 %) were moderate biofilm producers, and

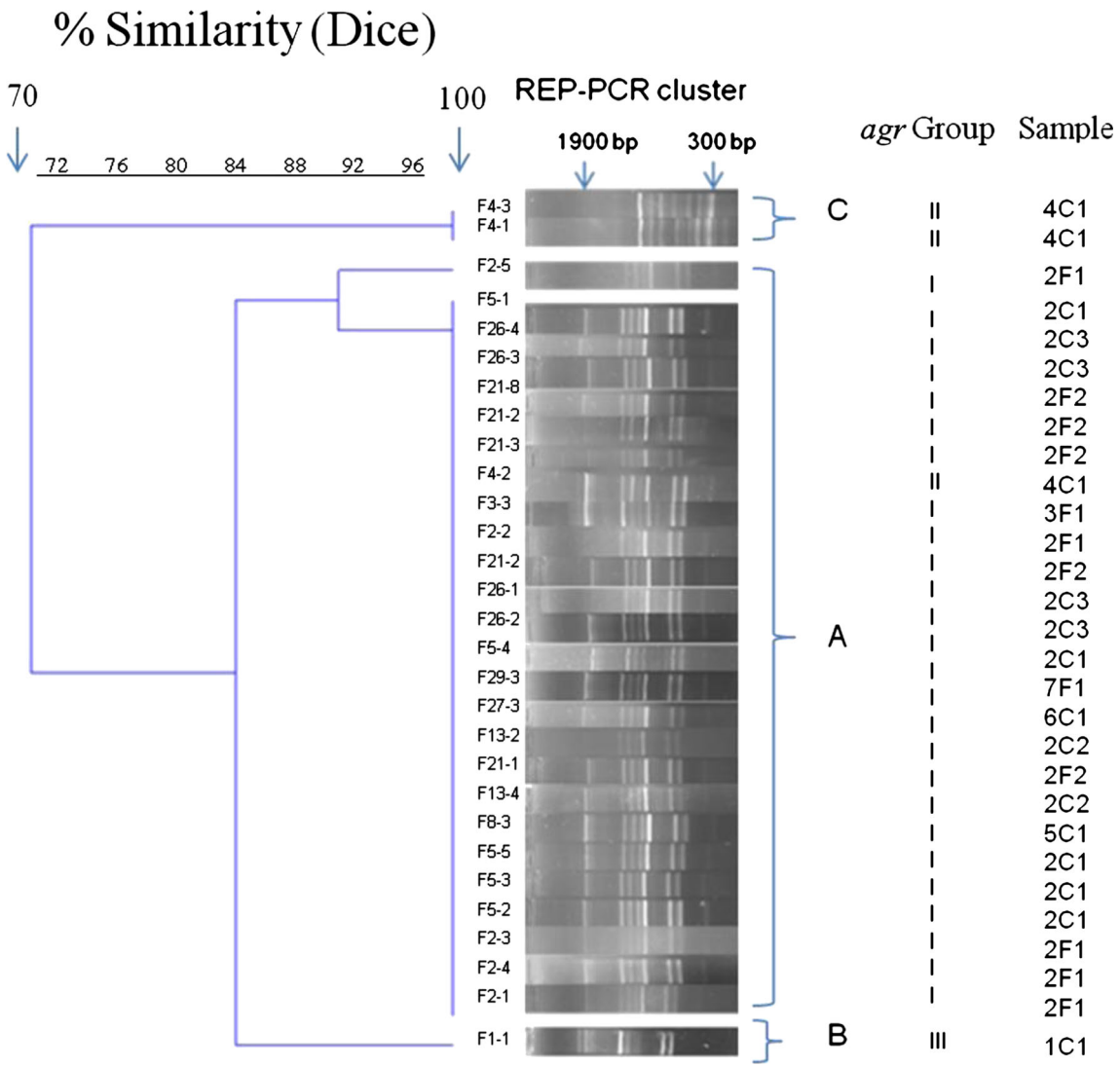

Fig. 2 Characteristic rep-PCR fingerprint patterns, clustering, and *agr* grouping by PCR-RFLP analysis of *S. aureus* isolated from raw poultry obtained from seven different Brazilian broiler chicken processors in South Brazil

2 (6.89 %) produced non-biofilm producers (Table 3). Rodrigues et al. (2010) found that all *S. aureus* isolates from a poultry slaughterhouse in southern Brazil were able to produce biofilm. There is widespread recognition that biofilm-forming pathogens are a real issue in the food processing industry. Biofilms are a persistent source of microbial contamination and can cause product spoilage, food safety problems, and loss of production efficiency (Jessen and Lammert 2003).

The *icaA* was the prevalent gene (93.10 %) in the isolates, and it was similarly common in all samples (Table 3), a finding that is consistent with other studies reporting a high frequency of the *icaA* gene in *S. aureus* isolated from food (Gutiérrez et al. 2012; Tang et al. 2013; Vázquez-Sánchez et al. 2013). The *sasG* gene was observed in all *S. aureus* strains isolated from raw poultry (Table 3). Tang et al. (2013) also observed that 100 % of *S. aureus* isolates from chicken samples associated with food poisoning and from slaughtered goats tested positive for the *sasG* gene. The presence of this gene in food may be related to handling procedures, as SasG is an adhesin that promotes bacterial adherence to nasal epithelial cells (Roche et al. 2003), and plays an important role in biofilm formation, facilitating adherence to host tissues. This study is the first to report the *sasG* gene in isolates from food in South America. Reiter et al. (2012) reported that the *sasG* gene was present in clinical isolates of *S. aureus* in South America. The *atlA* gene, which encodes an autolysin involved in the initial stages of biofilm formation, was present at a lower frequency (51.72 %) in *S. aureus* strains than the *sasG* and *icaA* genes (Table 3). Reiter et al. (2012), on the other hand, reported the presence of the *atlA* gene in all clinical *S. aureus* strains. This difference in prevalence may indicate that *atlA* is more common in clinical than food strains, but further studies would be required to confirm this.

Table 3 Distribution of virulence genes and biofilm ability in *Staphylococcus aureus* isolated from raw poultry obtained from seven different Brazilian broiler chicken processors in South Brazil

Sample	Isolates	Virulence genes identified by PCR	Biofilm formation
1 F1	F1-1	*icaA*, *atlA*, *sasG*	Non-biofilm
2 F1	F2-1, F2-3 F2-4	*icaA*, *sasG*	Strong
	F2-2	*icaA*, *atlA*, *sasG*	Strong
	F2-5	*icaA*, *sasG*	Moderate
2C1	F5-1, F5-2, F5-3	*icaA*, *sasG*	Strong
	F5-4	*icaA*, *sasG*	Strong
	F5-5	*icaA*, *atlA*, *sasG*	Strong
2C2	F13-2, F13-4	*icaA*, *atlA*, *sasG*	Strong
2 F2	F21-1, F21-3, F21-7, F21-8	*icaA*, *sasG*	Strong
	F21-2	*icaA*, *atlA*, *sasG*	Strong
2C3	F26-1	*sasG*	Moderate
	F26-2	*icaA*, *sasG*	Strong
	F26-3, F26-4	*icaA*, *atlA*, *sasG*	Moderate
3 F1	F3-3	*icaA*, *atlA*, *sasG*	Strong
4C1	F4-1, F4-2	*icaA*, *atlA*, *sasG*	Moderate
	F4-3	*atlA*, *sasG*	Non-biofilm
5C1	F8-3	*icaA*, *atlA*, *sasG*	Strong
6C1	F27-3	*icaA*, *atlA*, *sasG*	Moderate
7 F1	F29-3	*icaA*, *atlA*, *sasG*	Moderate

Hemolysin, protease, and lipase production of S. aureus

In the present study, 72.41 % (21/29) of *S. aureus* isolates demonstrated β-hemolytic activity (Table 4), a rate similar to that reported by Gundogan et al. (2012), who observed that 75.0 % of *S. aureus* isolated from meat and milk showed hemolytic activity. Hemolysin is an important virulence factor that has a potent toxic effect on lymphocytes, macrophages, neutrophils, epithelial cells, fibroblasts, and other cell lineages (Gundogan et al. 2013).

There have been fewer studies of protease and lipase activity in *S. aureus* isolated from raw chicken meat. We found that 62.06 % and 96.55 % of the *S. aureus* isolates displayed proteolytic and lipolytic activity, respectively (Table 4). Gundogan et al. (2013) reported that 80.0 % of *S. aureus* isolates obtained from chicken, meat, milk, and other food samples showed proteolytic activity, and 45.0 % displayed lipolytic activity. Takeuchi et al. (1999) reported that protease-positive strains of *S. aureus* were isolated from chickens suffering from dermatitis. We found that *S. aureus* isolates from raw poultry were lipolytic and proteolytic. Parkash et al. (2007) reported the sole lipolytic activity in *S. aureus* isolated from raw milk. Proteolytic and lipolytic bacteria are an issue in the food industry, as their proteases and lipases can cause food spoilage.

Table 4 Production of hemolysins, protease, and lipase in *Staphylococcus aureus* isolates from raw poultry in South Brazil

Sample	β-Hemolysins		Proteolysis		Lipolysis	
	n	%	n	%	n	%
1 F1	0	0	0	0	1	3.44
2 F1	3	10.34	4	13.79	4	13.79
2C1	4	13.79	5	17.24	5	17.24
2C2	2	6.89	0	0	2	6.89
2 F2	5	17.24	3	10.34	5	17.24
2C3	5	17.24	3	10.34	4	13.79
3 F1	0	0	0	(0	1	3.44
4C1	0	0	1	3.44	3	3.44
5C1	0	0	0	0	1	3.44
6C1	1	3.44	1	3.44	1	3.44
7 F1	1	3.44	1	3.44	1	3.44
Total	21	72.41	18	62.06	28	96.55

Conclusions

The evaluation by rep-PCR of *S. aureus* isolates from raw poultry obtained from various Brazilian broiler chicken processors suggests that there may be clonal relationships among the strains that may be related to the selection of resident strains adapted for colonization in food processing facilities. We found that most isolates belonged to *agr* group I. The presence of biofilm-forming *S. aureus* strains in raw poultry, their ability to harbor biofilm-related genes, and the spoilage features that they exhibit are indicative of their high pathogenic potential that may represent a serious issue for the food processing industry.

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