Dairy Sci. & Technol.
DOI 10.1007/s13594-014-0195-0

ORIGINAL PAPER

Molecular characterization and antimicrobial susceptibility of *Staphylococcus aureus* from small-scale dairy systems in the highlands of Central México

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Received: 27 May 2014 / Revised: 10 September 2014 / Accepted: 11 September 2014

Abstract *Staphylococcus aureus* is involved in human and animal infections. Because of mastitis in dairy cows, milk can be contaminated by enterotoxin-producing strains, which constitutes a food poisoning risk. Animal handlers can be asymptomatic carriers, becoming an additional source of contamination. This research aims to improve our understanding of *Staphylococcus aureus* in small-scale dairy systems in central Mexico. Samples were taken in 12 dairy farms and included composite milk (from the four teats) and hand swabs from

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Published online: 01 October 2014

each farmer. Of 149 presumptive *S. aureus* isolates, 102 (84 from cows; 18 from farmers) were accurately identified by molecular methods. The genetic variability of 43 randomly selected isolates was determined by RAPD-PCR, and of these, 38 were genetically distinct (≤90% similarity). Of the 38 distinct isolates, 78.9% harboured at least one enterotoxin-encoding gene (*staphylococcal enterotoxin a* (*sea*)–*sed*, *seg*, and/or *sei*), whereas 100% carried *icaA–icaD* genes and 28% carried the *bap* gene. The latter three genes are all involved in biofilm formation. Susceptibility to antibiotics, bacteriocins, and bacteriophages, was also assayed; 36.8% of isolates were resistant to penicillin G. Only five isolates were resistant to bacteriocins nisin A and AS-48, and phiPLA-RODI was the most effective bacteriophage, infecting all of the isolates. These results support the need to adopt management strategies to improve hygiene and milking practices in order to enhance herd health and diminish the risk of food poisoning associated with the consumption of raw milk cheese.

Keywords *Staphylococcus aureus* · Enterotoxins · Biofilms · Antimicrobials · Small-scale dairy systems · Mexico

1 Introduction

Small-scale dairy systems (SSDS) provide an option for improving rural livelihoods and alleviating poverty (Espinoza-Ortega et al. 2007). In Mexico, over 78% of specialized dairy farms are small scale, defined by small holdings, with herds of between 3 and 35 cows plus replacements, that rely on family labour, and these systems contributed 37% of national milk production (Hemme et al. 2007). Milk hygiene is a challenge for these small-scale dairy systems involving good milking practices and both animal and human health.

Staphylococcus aureus is a coagulase-positive microorganism frequently detected in dairy farms and considered one of the main causes of sub-clinical and chronic mastitis in dairy cows (Makovec and Ruegg 2003; Wang et al. 2009), with negative effects in the economics of milk production. Cattle are one of the primary reservoirs for staphylococci, and an udder with mastitis is usually the main source of contamination by *S. aureus* (Waldvogel 2001). Humans can be asymptomatic carriers and, consequently, a source of contamination too (Gutiérrez et al. 2012; Rall et al. 2014). Moreover, some strains produce heat-stable enterotoxins and are responsible for food poisoning (Le Loir et al. 2003). The ability of *S. aureus* strains to produce biofilms is suggested as a virulence factor associated to staphylococcal mastitis and accounts for the establishment of chronic infections as a reduced susceptibility to antibiotics and disinfectants occurs due to the decreased diffusion of antimicrobials through the biofilm's matrix (Vasudevan et al. 2003). Therefore, strains bearing biofilm-encoding genes have an additional, potential pathogenicity that must be taken into account.

Due to the significant impact on animal and human health, it is important to assess the resistance of *S. aureus* isolates to antibiotics since antibiotic therapy is the primary means of combating bacterial infections. However, the detection of multidrug-resistant *S. aureus* is increasingly common, and reduced efficacy of antibiotic therapy has become a matter of concern in veterinary practice (Lee 2003). In this regard, there is a clear need for alternative approaches to mastitis treatment such as bacteriocins (Cao et al. 2007) and bacteriophages (Dias et al. 2013). Bacteriocins are bacterial ribosomally synthesized peptides with antimicrobial activity. Apart from their role as biopreservatives (Deegan et al. 2006), some bacteriocins produced by lactic acid bacteria have been successfully

assayed against pathogens causing bovine mastitis (Klostermann et al. 2010). Bacteriophages, natural enemies of bacteria, have also been tested to combat human (Duckworth and Gulig 2002) and bovine infections (Gill et al. 2006; Dias et al. 2013).

The present work aimed to evaluate the prevalence of *S. aureus* in a sample of small-scale dairy systems located in the highlands of Central Mexico in order to be able to suggest ways of improving herd health and the microbiological quality of milk since this is used in the manufacture of raw milk cheeses with the concomitant risk for consumers. Putative virulence determinants such as enterotoxin-encoding genes and the biofilm-forming ability of staphylococcal isolates were analyzed. Susceptibility to antibiotics and natural antimicrobials such as nisin A (lantibiotic) and AS-48 (circular bacteriocin) and *S. aureus*-infecting bacteriophages was approached. Additionally, the genetic variability and relationships among the isolates were established by random amplified polymorphic DNA (RAPD)-PCR.

2 Material and methods

2.1 Sampling design

Twelve small-scale dairy farms in the municipality of Aculco in the State of Mexico were selected from a larger sample of farms participating in a larger project (Fadul-Pacheco et al. 2013), as representative of farms of this Mexican area. Criteria for selection were all cows of the same breed (Holstein—being the most prevalent), a minimum of three milking cows at the time of the study (a maximum of five cows per farm were sampled) and hand milking. Age and stage of lactation were not considered or recorded. Sampling took place between November 2012 and January 2013 always during milking. A total of 57 composite milk samples were obtained by mixing milk from the four quarters (one sample per cow) after the udders had been washed and dried and the teats had been cleaned with cotton swabs soaked in 70% ethanol. Milk from the first streams was included in the sample in order to isolate environmental microorganisms that could be present at the sphincter, as well as those from the mammary gland. Additionally, 12 samples were taken from the hands of the 12 farmers using a sterile swab and placing it into 3 mL of nutritive broth. All samples were kept at 4 °C until processing in the laboratory.

2.2 Isolation and identification of *S. aureus* strains

Serial tenfold dilutions of milk samples were made in Ringer solution (Merck KGaA, Darmstadt, Germany), and 0.1 mL of each was plated onto Baird–Parker (BP) agar supplemented with egg yolk tellurite emulsion (Scharlab, Barcelona, Spain) in order to obtain single colonies for further characterization. Undiluted hand swabs were also spread onto BP agar. Each sample was plated in duplicate, and all plates were incubated at 37 °C for 48 h.

Ten colonies showing the typical aspect of staphylococci were randomly selected from each plate and spread onto salt-mannitol agar to obtain pure cultures, and these were tested for coagulase (Bactident Coagulase, Merck), DNase activity (DNase agar, Cultimed, Panreac, Barcelona, Spain) and mannitol fermentation under anaerobiosis (Notarnicola et al. 1985). All presumptive *S. aureus* isolates were further confirmed by simultaneous PCR amplification of genes encoding for 23S ribosomal RNA (rRNA) (1,250 bp) (Straub et al. 1999) and thermonuclease (*nuc*) (450 bp) (Wilson et al. 1991)

(Table 1). Isolates were coded according to their origin (the prefix M for milk and H for hand swabs). *S. aureus* Sa9, isolated from mastitis milk (García et al. 2007), and *S. aureus* ATCC 14458 were used as positive controls; *Staphylococcus epidermidis* F12 was used as a negative control (Delgado et al. 2009). All isolates were routinely cultured in tryptic soy broth (TSB, Scharlab) or in tryptic soy agar (TSA). Bacterial stocks were stored at −80 °C in TSB supplemented with 20% *v*/*v* glycerol.

2.3 DNA extraction and PCR conditions

A colony from each presumptive *S. aureus* isolate was added to 20 μL of 0.25% SDS–50 mM NaOH and heated at 95 °C for 5 min. Deionized water was subsequently added up to a volume of 200 μL. Samples were then centrifuged at 13,000 rpm for 5 min at 4 °C, and the supernatant containing the DNA was kept at −20 °C for further analysis.

All PCRs were performed in a thermocycler (Bio-Rad, Hercules, CA, USA) using the Pure Taq Ready-To-Go PCR Beads kit (GE Healthcare, Munich, Germany), 1 μL of the extracted DNA and the primer concentrations specified in Table 1. The PCR conditions specified in Table 1 were used, with the exception of those used for the detection of the 23S RNA and the *nuc* genes, where a multiplex PCR was performed composed of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 s, 53 °C for 1 min and 72 °C for 2 min and eventually a final extension step at 72 °C for 7 min.

PCR products were loaded onto 2% agarose gels and electrophoresis carried out for 1 h at 100 V in Tris–acetate–EDTA. The gels were then stained with ethidium bromide (0.5 $\mu g.mL^{-1}$) for 30 min and visualized under UV light with a G: Box Syngene™ transilluminator (Syngene, Cambridge, UK). DNA ladders of 500 or 100 bp were included in the gels as molecular weight controls.

2.4 Genomic fingerprinting of *S. aureus* isolates

The RAPD-PCR method was chosen for the differentiation of the *S. aureus* isolates. Primers used for this purpose were OPL5, RAPD5 and P1 (Table 1) according to the method previously described (Gutiérrez et al. 2011). Strains of *S. aureus* from the *Instituto de Productos Lácteos de Asturias—Consejo Superior de Investigación Científica* (IPLA-CSIC—Dairy Institute of Asturias, Spain) and *S. aureus* ATCC 14458 were included to enable the comparison of genetic variability among Mexican *S. aureus* and isolates from distant geographical locations.

RAPD-PCR band patterns from each primer were scanned and profile grouping (dendrogram) performed with the GENTOOLS Syngene™ software (Syngene, Cambridge, UK), using the Pearson product-moment correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis (Struelens 1996).

2.5 Detection of putative virulence factors

The presence of genes encoding for two virulence factors (enterotoxin and biofilm production) in the genome of the staphylococcal isolates was tested. The detection of eight enterotoxin genes was performed using two multiplex PCRs. The first included the *staphylococcal enterotoxin a* (*sea*), *seb*, *sec*, *sed* and *see*-specific primers, while the second used primers to detect the *seg*, *seh* and *sei* genes (Table 1). Additionally, assessment of the

Table 1 Primers and bacterial strains used in the PCR (adapted from Gutiérrez et al. 2012)

Gene	Control, *S. aureus* strain	Primer sequence (5′–3′)	Final concentration for PCR (μM)	Amplicon size (bp)	Reference
23S rRNA	Sa9	ACGGAGTTACAAAGGACGAC	0.2	1,250	Straub et al. (1999)
		AGCTCAGCCTTAACGAGTAC			
nuc	Sa9	AGTATATAGTGCAACTTCAACTAA	0.8	450	Wilson et al. (1991)
		ATCAGCGTTGTCTTCGCTCCAAAT			
RAPD-PCR		OPL5: ACGCAGGCAC	1		Gutiérrez et al. (2011)
		RAPD5: AACGCGCAAC			
		P1: CCGCAGCCAA			
sea[a]	ATCC 13565	CCTTTGGAAACGGTTAAAACG	10	127	Becker et al. (1998)
		TCTGAACCTTCCCATCAAAAAC			
seb[a]	ATCC 14458	TCGCATCAAACTGACAAACG	10	477	
		GCAGGTACTCTATAAGTGCCTGC			
sec[a]	ATCC 14458	CTCAAGAACTAGACATAAAAGCTAGG	0.4	271	
		TCAAAATCGGATTAACATTATCC			
sed[a]	ATCC 13565	CTAGTTTGGTAATATCTCCTTTAAACG	10	319	
		TTAATGCTATATCTTATAGGGTAAACATC			
see[a]	ATCC 27664	TAACTTACCGTGGACCCTTC	10	178	
		CAGTACCTATAGATAAAGTTAAAACAAGC			
sea[a]	ATCC 19095	AAGTAGACATTTTTGGCGTTCC	2	287	Omoe et al. (2002)
		AGAACCATCAAACTCGTATAGC			
seh[a]	ATCC 19095	GTCTATATGGAGGTACAACACT	2	213	
		GACCTTTACTTATTTCGCTGTC			
set[a]	ATCC 19095	GGTGATATTGGTGTAGGTAAC	2	454	
		ATCCATATTCTTTGCCTTTACCAG			

Table 1 (continued)

Gene	Control, *S. aureus* strain	Primer sequence (5′–3′)	Final concentration for PCR (μM)	Amplicon size (bp)	Reference
icaA	ATCC 15981	CCTAACTAACGAAAGGTAG AAGATATAGCGATAAGTGC	2.5	1,315	Vasudevan et al. (2003)
icaD	ATCC 15981	AAACGTAAGAGAGGTGG GGCAATATGATCAAGATAC	2.5	381	
Bap	V329	CCCTATATCGAAGGTGTAGAATTGCAC GCTGTTGAAGTTAATACTGTACCTGC	2.5	971	Cucarella et al. (2004)

[a] Primers were grouped in the same multiplex PCR reaction

[b] Primers were grouped in the same multiplex PCR reaction

capability to form biofilms was performed using two further multiplex PCRs. The first combined specific primers to detect simultaneously the *icaD* and biofilm-associated protein (*bap*) genes, and the second used only the *icaA* primer (Table 1).

2.6 Susceptibility to antibiotics

Antibiotic susceptibility of staphylococcal isolates was determined by the disc diffusion method according to the CLSI (2010). The following discs were provided by Oxoid (Basingstoke, UK): ciprofloxacin (CIP5), enrofloxacin (ENR5), erythromycin (E15), oxacillin (OX5), penicillin G (P10), quinupristin–dalfopristin (QD15), rifampicin (RD5), streptomycin (S10), sulphamethoxazole–trimethoprim (SXT25) and tetracycline (TE30). They are used as antimicrobial agents for food-producing animals (OIE 2014; Commission Regulation (EU) No 37/ 2010) and are representative of different classes of antibiotics. Vancomycin (VA5), which belongs to the glycopeptide class, a critically important antibiotic used in human medicine, was also tested (CIA 2011). Oxacillin was included for the detection of putative methicillin-resistant isolates (CLSI 2010). Plates of Mueller-Hinton agar (Scharlab) seeded with each staphylococcal isolate were incubated at 37 °C for 24 h and the inhibition zone diameters interpreted according to the CLSI tables. The minimum inhibitory concentration (MIC) of isolates resistant to penicillin G was determined by using the MIC Evaluator (M.I.C.E.)^{TM} (32–0.002 $\mu g.mL^{-1}$) MA0100D system (Oxoid). *S. aureus* ATCC 25923 was used as a positive control for the inhibition halo formation.

2.7 Susceptibility to bacteriocins

The antimicrobial activity of bacteriocins against the staphylococcal isolates was determined by the agar diffusion test. For this purpose, pure nisin A (kindly supplied by Applin & Barret Ltd, Dorset, UK) and AS-48 (kindly supplied by Dr. Mercedes Maqueda, University of Granada, Spain) were used. The concentration of stock solutions for nisin A and AS-48 were 30 and 35 μM, respectively. Twenty millilitres of TSA (1.2% *w/v* agar) was inoculated with staphylococcal isolates at ca. 10^5 colony forming units (CFU) mL^{-1} and pour plated. Wells (4 mm in diameter) were made with a sterile borer and filled with twofold dilutions of bacteriocin solutions in phosphate-buffered saline solution. MIC was defined as the lowest concentration that produced a clear inhibition halo after incubation at 37 °C for 24 h (Martínez et al. 2005). The range of bacteriocin concentrations assayed was the following: nisin A (30, 15, 7.5, 3.75, 1.875, 0.937 and 0.468 μM) and AS-48 (35, 17.5, 8.75, 4.375, 2.187, 1.093 and 0.547 μM). *S. aureus* CECT 4013 was used as positive control for the inhibition halo formation.

2.8 Susceptibility to bacteriophages

The IPLA-CSIC collection bacteriophages vB_SauS-phiIPLA35 (in short, phiIPLA35) and vB_SauS-phiIPLA88 (in short, phiIPLA88) (García et al. 2007), vB_SauM-phiIPLA-RODI (in short, phiIPLA-RODI) and vB_SepM-phiIPLA-C1C (in short, phiIPLA-C1C) (Gutiérrez et al., unpublished data) and phi11 (Iandolo et al. 2002) were used to determine their ability to lyse the staphylococcal isolates. The susceptibility of isolates to the phages was determined by the spot test. Five microlitres of each

phage (phage titre about 10^9 PFU.mL^{-1}) was dropped onto a TSA plate overlaid with about 10^6 CFU.mL^{-1} of each isolate (Gutiérrez et al. 2010). After incubation at 37 °C for 24 h, the presence of a clear lysis zone is representative of phage susceptibility.

3 Results

3.1 Isolation and identification of *S. aureus*

From a total of 149 presumptive *S. aureus* isolates found in the 12 farms (from milk to hand swabs), 102 (84 from milk and 18 from hand swab samples) were identified as *S. aureus* according to biochemical methods. The positive identification was confirmed by the detection of specific amplicons corresponding to the 23S rRNA and *nuc* genes. The positive isolates were detected in 30 of 57 cows (52.6%) and in 8 of 12 hand swabs (66.6%). *S. aureus* was not detected in the milk from two of the farms (6 and 12), and four cattle handlers were not *S. aureus* carriers (farms 4, 10, 11 and 12).

3.2 Genetic variability of *S. aureus* isolates

The genetic variability of 43 positive isolates (one for each milk sample and one for each hand swab, randomly selected) was determined by RAPD-PCR genotyping using three different primers (Table 1). Isolates were grouped into four main clusters (I–IV). With the exception of cluster II, the others were further divided into eight sub-clusters (Ia, Ib; IIIc, IIId; IVe, IVf, IVg, IVh) (Fig. 1). Cluster I, with 37% similarity with clusters II, III and IV, is composed of isolates from four different farms. Cluster II, with 43.5% of similarity with clusters III and IV, includes two strains with very different origin (M16, a Mexican isolate and the collection strain ATCC 14458). Of note, cluster III, with 47.05% similarity with cluster IV, includes six of the eight hand isolates and only one milk isolate from a different farm (farm 4). Group IV comprises most of isolates (28 from milk and 2 from hand swab samples) that were grouped into four sub-clusters each belonging to a different farm. Only one Mexican strain (M31) fell completely outside of the four clusters since it is more closely related to the Spanish isolate IPLA 18 (52% similarity) than to any of the other Mexican isolates. The Spanish isolates Sa9 and IPLA 1 are also outside of the clusters due to their low similarity (32.5 and 38.6%, respectively) with the other isolates.

For discriminating strains, a threshold similarity value of 90% was set, so that isolates with a similarity above this value were considered clonal and excluded from further analyses (M2, M8, M9, M11, and M28). Accordingly, the 43 Mexican isolates included in the dendrogram (Fig. 1) showed 38 different RAPD-PCR profiles (30 from milk and 8 from hand swabs).

3.3 Putative virulence factors of *S. aureus* isolates

One isolate representative of each of the 38 different RAPD-PCR profiles was selected and tested for the presence of enterotoxin and biofilm-encoding genes. The most widely distributed genes were *sea* (47.3%), *seg* (55.2%) and *sei* (31.5%), while *seb* and *sec* genes were only detected in one unique isolate (M16 and M35, respectively), and no isolate harboured either *seh* or *see* genes (Fig. 2a). Overall,

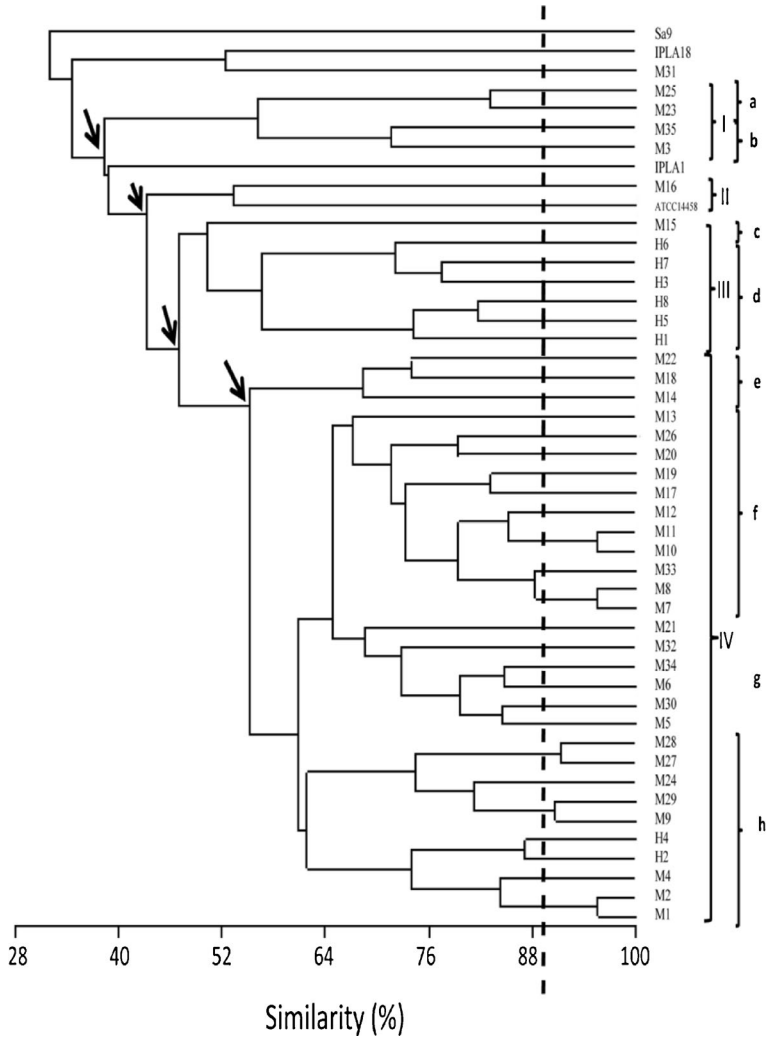

Fig. 1 *Arrows* indicate the similarity percentage arbitrarily chosen as a discriminating threshold to define the homogenous clusters (I–IV). The *dashed line* represents the threshold similarity value of 90 % for discriminating genetically different strains

30 of 38 isolates (78.9%) harboured between one and five enterotoxin-encoding genes (*sea*, *seb*, *sec*, *sed*, *seg* and *sei*) with the following distribution: one gene (eight M and four H isolates), two genes (six M and two H isolates), three genes (five M and one H isolates), four genes (one M isolate) and five genes (one M isolate).

In relation to biofilm production genes, all the isolates tested positive for the *icaA* and *icaD* genes, which are responsible for the formation of an exopolysaccharide biofilm matrix, whereas the *bap* gene, responsible for the synthesis of a bacterial surface protein involved in the formation of a proteinaceous biofilm matrix, was detected in 11 of the 38 isolates (28.9%) belonging to six farms (2, 3, 4, 5, 8 and 11). It is worth noting that only one of the isolates (H7, farm 8) harbouring the *bap* gene was of human origin (Fig. 2b).

3.4 Antimicrobial susceptibility

3.4.1 Antibiotics

Fourteen of the 38 isolates (36.8%) showed resistance to penicillin G (P10). One of them (M15) also showed resistance to streptomycin (S10). Resistance to tetracycline (TE30) was observed in isolate M17, and intermediate resistance to erythromycin (E15) was shown by the milk isolate M7 (Table 2). The MICs of penicillin-G-resistant isolates ranged from 0.25 to 0.5 $\mu g.mL^{-1}$ regardless of their origin (milk or cattle handler), with clearly higher resistance than that shown by the control strain *S. aureus* ATCC 25923 (0.03 $\mu g.mL^{-1}$).

3.4.2 Bacteriocins

The susceptibility of *S. aureus* isolates to the lantibiotic nisin A and the circular bacteriocin AS-48 was determined. The MICs of nisin and AS-48 for most of the strains were 7.5 and 8.75 μM, respectively, suggesting that both bacteriocins displayed similar antimicrobial potency against these isolates (Table 2). Furthermore, both bacteriocins were active against *S. aureus* strains, regardless of whether they were isolated from milk or cattle handlers, and both populations showed a similar MIC distribution. Nevertheless, resistant strains to the highest bacteriocin concentrations were detected. Of note, the human isolates H1 and H8 were resistant to both bacteriocins. The control strain *S. aureus* CECT 4013 showed an MIC of 7.5 μM for nisin as did most of isolates, but its MIC was clearly higher for AS-48 (35 μM).

3.4.3 Bacteriophages

The susceptibility of *S. aureus* isolates to bacteriophages from the IPLA-CSIC collection was assessed (Table 2). All 38 isolates were sensitive to phage vB_SauM-phiIPLA-RODI but resistant to phage vB_SauM-phiIPLA-C1C. The lytic activity of phages vB_SauS-phiIPLA88 and phi11 was very similar, showing almost identical host inhibition spectra (32 and 33 isolates, respectively). Two of the isolates that were resistant to both phages are of human origin.

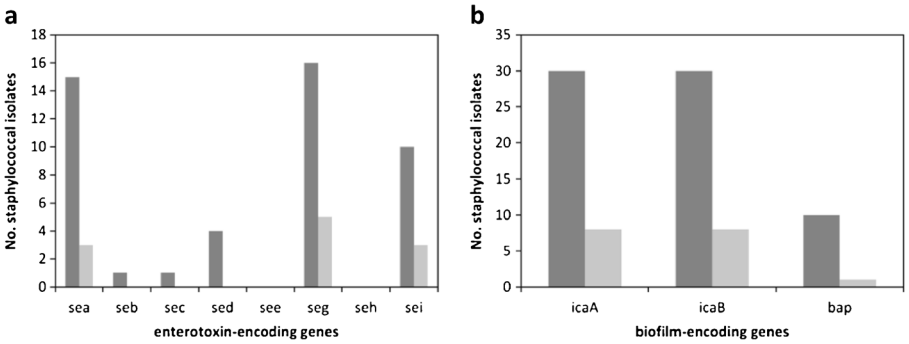

Fig. 2 Putative virulence factors in *S. aureus* isolates from Mexican small-scale dairy systems. **a** Number of isolates harbouring *se* genes; **b** number of isolates harbouring biofilm-encoding genes. Dark grey bars: isolates from milk; Light grey bars: isolates from hand swabs

Table 2 Antimicrobial susceptibility of the 38 genetically different *S. aureus* isolates from Mexican cow's farms

	Number of resistant isolates										
Antibiotics (disc diffusion method)	CIP5	ENR5	E15	OX5	P10	QD15	RD5	S10	SXT25	TE30	VA5
Isolates from milk (n=30)	0	0	1[a]	0	10	0	0	1	0	1	0
Isolates from handlers (n=8)	0	0	0	0	4	0	0	0	0	0	0
MICs (μM) for bacteriocins (agar diffusion test)	Nisin A >30	30	15	7.5	3.75		AS-48 >35	35	17.5	8.75	4.375
Isolates from milk (n=30)	2	2	3	14	9				8	14	8
Isolates from handlers (n=8)	2		2	3	2		1	1	3	1	2
S. aureus CECT 4013: MIC for nisin A of 7.5 μM; MIC for AS-48 of 35 μM											
Host inhibition spectrum of bacteriophages	phi11		phiIPLA88		phiIPLA35		phiIPLA-RODI		phiIPLA-C1C		
Isolates from milk (n=30)	27		26		13		30		0		
Isolates from handlers (n=8)	6		6		1		8		0		
S. aureus Sa9 (phage indicator strain): susceptible to phages phi11, phiIPLA88, phiIPLA35; phiIPA-RODI; *S. aureus* IPLA 16 susceptible to phiIPLA-C1C											

[a] Intermediate resistance

All of the isolates showed greater variability in relation to their susceptibility to phage vB_SauS-phiIPLA35. Fourteen of the isolates (36.8%) were sensitive, but most of the human isolates (seven of eight) were resistant to this phage.

4 Discussion

The prevalence of *S. aureus* in small farms in the highlands of Central Mexico was investigated as this microorganism is a common cause of udder disease in dairy cows and has implications for human health. Milk samples from 30 of 57 cows (52.6%) and 8 of 12 hand swabs (66.6%) tested positive for *S. aureus*. The pathogen was detected neither in milk from two of the farms (6 and 12) nor on hand swabs from four of the cattle handlers (farms 4, 10, 11 and 12). In comparison, *S. aureus* prevalence was substantially lower in milk samples (6.6%) from dairy farms of a similar size in the state of Sao Paolo, Brazil (Lee et al. 2012).

Forty-three isolates, representative of each positive sample, were subjected to RAPD-PCR genotyping to define their genetic diversity. The cluster analysis revealed four different clusters (I–IV), and 38 isolates were identified as genetically distinct (≤90% similarity). This study revealed a weak relationship between milk and human isolates, suggesting that transmission of *S. aureus* from farmers to cows has not yet occurred on the farms included in this study. By contrast, a study of dairy farms in Central-Eastern Mexico (Manjarrez-López et al. 2012) reported 79.3% of *S. aureus* isolates showing human origin, indicating the importance humans as vectors for *S. aureus* transmission. Data supporting animals and humans as sources of *S. aureus* zoonotic infections of humans and pathogens infecting livestock have both previously been reported (Van Cleef et al. 2011; Sakwinska et al. 2011).

RAPD-PCR genotyping showed certain variability among the isolates from milk samples. Most of them belong to cluster IV and are distributed among four sub-clusters (IVe–IVh). The three isolates from farm 1, however, are grouped into only one sub-cluster (IVh). Cluster I harbours isolates from four different farms. A similarity exceeding 90% was only detected between isolates within farms 1, 2 and 3. By contrast, human isolates showed lower variability. Six of eight isolates belong to sub-cluster IIId, with a similarity ranging from 56 to 81%. The remaining isolates (H2 and H4, sub-cluster IVh), with 87% similarity, are closer to isolates from farm 1 (74% similarity) than to any of the others. As expected, strains from distant geographical locations (Sa9, IPLA 1, IPLA 18 and ATCC 14458) showed very low genetic relationship with Mexican isolates, with the exception of isolates M31 and M16.

The presence of enterotoxin-producing and biofilm formation genes in the genome of *S. aureus* isolates contribute to their pathogenicity. Most of the 38 distinct isolates are multi-SE gene carriers (73.7%) and, consequently, potentially enterotoxigenic and were detected in the ten farms that tested positive for *S. aureus*. The non-enterotoxigenic isolates (23.3% from milk samples) are distributed among four farms (5, 7, 9 and 11). Interestingly, the unique isolate from human origin (H3) that does not harbour any *se* genes was detected in a different farm (farm 3). The presence of the *sea* gene in most of the milk isolates from farms 1, 2, 3, 4 and 5 makes them a potential risk for consumers, as SEA is the most common enterotoxin in staphylococcal food poisoning (Pinchuk et al. 2010). The *sea* gene was also detected in three isolates from

cattle handlers (farms 7, 8 and 9). Thus, the use of raw milk from the majority of the farms studied carries a health risk for consumers.

The putative pathogenicity of isolates is increased by their ability to produce biofilms (Valle et al. 2012). All of the isolates are potential polysaccharide-type biofilm producers as they carry *icaA* and *icaD* genes involved in the biosynthesis of polysaccharide intracellular adhesion (PIA) (Crampton et al. 1999). Moreover, the *bap* gene that is related to the formation of proteinaceous biofilms (Cucarella et al. 2001) was also detected in ten milk isolates and one human isolate (H7, farm 8). This is particularly relevant because, so far, this gene had been only associated with the infection of bovine udders (Cucarella et al. 2004), meanwhile *bap*-harbouring strains associated with human infections are coagulase negative *Staphylococcus* (Arciola et al. 2012). None of the milk isolates from farm 8 is, however, a *bap* gene carrier.

The susceptibility to antibiotics tests are an important guide for veterinarians to select the most suitable treatment when udder infection occurs. In this context, the sensitivity of the isolates to ten antibiotics approved for veterinary use, and to vancomycin, used in human medicine, was determined. Only resistance to penicillin was particularly relevant, being detected in 33.3% (10/30) of milk isolates (farms 1, 4, 5, 7, 8, 9 and 10) and in half the human isolates (Farms 2, 3, 5 and 7), giving an overall resistance of 36.8% (14/38). Of those, only one isolate (M15) was also resistant to streptomycin. The great concern about MRSA strains (Lee 2003) makes it especially relevant that none of the isolates in the present study was resistant to oxacillin. The pattern of antibiotic resistance identified in this study suggests that penicillin is the most frequently used antibiotic to treat mastitis.

Nisin A and AS-48 bacteriocins were chosen as antimicrobial alternatives to antibiotic therapy (Cotter et al. 2013) due to their proved activity against *S. aureus* (Delves-Broughton et al. 1996; Sánchez-Hidalgo et al. 2011). Most isolates were sensitive to the bacteriocin concentrations tested. This supports their potential use to fight against the staphylococcal isolates that colonize the small dairy farms analyzed in this study. Two isolates from human origin (H1 and H8) showed resistance to 30 μM of nisin A. H8 was also resistant to 35 μM of AS-48. Whether they share the same resistance mechanism against nisin and AS-48 has not been addressed. However, it is likely that changes at the surface of H1 and H8 may reduce the initial electrostatic interactions between bacteriocins and cells and result in cross resistance to both cationic peptides as previously described (Kaur et al. 2011).

The sensitivity of the staphylococcal isolates was also tested against members of two phage families: *Siphoviridae* (phi11, phiIPLA88 and phiIPLA35) and *Myoviridae* (phiIPLA-RODI and phiIPLA-C1C). PhiIPLA-RODI was the most effective bacteriophage as it was able to infect and lyse all the isolates, regardless of their origin (bovine or human). Similar results were observed by Dias et al. (2013) since four of ten bacteriophages isolated from mastitic milks were effective against all the mastitis-causing *S. aureus* isolates. Phages phiIPLA88 and phi11 also showed notable antistaphylococcal effectiveness (84.2 and 86.8% of isolates) with almost identical host inhibition spectra. This result could be related to the high degree of genome identity (75.3%) and the notable shared protein content (65%) (García et al. 2009). A small host inhibition spectrum was shown by phage phiIPLA35, and most of resistant isolates are grouped in cluster III to which most human isolates belong (only one was sensitive). However, no human isolates were resistant to phage RODI, whereas two were resistant

to IPLA 88 and phi11. By contrast, phage phiIPLA-C1C was unable to infect any isolate. This result is in agreement with previous data (Gutiérrez et al., unpublished data) since this phage is much more active against *S. epidermidis* than against *S. aureus* strains. As a whole, these results support bacteriophages as a promising alternative to fight against antibiotic-resistant pathogens from human or bovine origin (Duckworth and Gulig 2002; Dias et al. 2013).

5 Conclusion

Overall, results highlight the need to improve hygiene conditions and milking practices in the small-scale dairy systems that were the focus of this study since potential enterotoxigenic and biofilm *S. aureus* producers are widely distributed in the farms. There is no evidence of *S. aureus* transmission between farmers and cattle since RAPD-PCR profiles of isolates from milk and human origin show low similarity. The resistance to penicillin G showed by 36.8% of isolates is particularly noteworthy and points to an antibiotic therapy against udder infection mainly based on penicillin treatment. Alternative approaches based on bacteriocins and bacteriophages, natural antimicrobials, have proved to be useful as potential tools to eliminate the pathogen from cattle and handlers in an environmentally friendly way, with the final aim of enhancing herd health and diminishing the inherent food poisoning risk associated with the consumption of cheese made with raw milk produced in these farms.

Acknowledgments The authors express their gratitude to the farmers and their families participating in this study. Also, our thanks are given to R. Calvo from IPLA-CSIC for her knowledge and technical support, as well as to L.E. Contreras-Martínez and M.L. Maya Salazar from ICAR for their laboratory support. We thank Dr. Graham Woodgate (University College London) for revising the English and making helpful suggestions for improving the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Funding This work was undertaken with funds from the Spanish Agency for International Development Cooperation (*Agencia Española de Cooperación Internacional para el Desarrollo*) through project AECID 11-CAP2-1526 and the Mexican National Council for Science and Technology (*Consejo Nacional de Ciencia y Tecnología—CONACYT*) grant 129449 CB-2009. CONACYT also provided the grant that enabled the postgraduate studies of Tania Berenice Salgado-Ruiz.

References

Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW (2012) Biofilm formation in staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials 33:5967–5982

Becker K, Roth R, Peters G (1998) Rapid and specific detection of toxigenic *staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. J Clin Microbiol 36:2548–2553

Cao LT, Wu JQ, Xie F, Hu SH, Mo Y (2007) Efficacy of nisin in treatment of clinical mastitis in lactating dairy cows. J Dairy Sci 90:3980–3985

CIA (2011). Critically Important Antimicrobials for Human Medicine. 3rd Revision. WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). WHO, Geneva

CLSI (2010) Performance standards for antimicrobial susceptibility testing; 20th international supplement. CLSI document M100-S20. Clinical and Laboratory Standards Institute Wayne, PA, USA

Commission Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L15/1-L15/72

Cotter PD, Ross RP, Hill C (2013) Bacteriocins - a viable alternative to antibiotics? Nat Rev Microbiol 11:95–105

Crampton SE, Gerke C, Schnell NF, Nichols WW, Gotz F (1999) The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun 67:5427–5433

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR (2001) Bap a, *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol 183:2888–2896

Cucarella C, Tormo MA, Ubeda C, Trotonda MP, Monzon M, Peris C, Amorena B, Lasa I, Penadés JR (2004) Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect Immun 72:2177–2185

Deegan LH, Cotter PD, Hill C, Ross P (2006) Bacteriocins: biological tools for bio-preservation and shelf-life extension. Int Dairy J 16:1058–1071

Delgado S, Arroyo R, Jiménez E, Marín ML, del Campo R, Fernández L, Rodríguez JM (2009) *Staphylococcus epidermidis* strains isolated from breast milk of women suffering infectious mastitis: potential virulence traits and resistance to antibiotics. BMC Microbiol 9:82

Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996) Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek 69:93–202

Dias RS, Eller MR, Duarte VS, Pereira AL, Silva CC, Mantovani HC, Oliveira LL, Silva E, De Paula SO (2013) Use of phages against antibiotic-resistant *Staphylococcus aureus* isolated from bovine mastitis. J Anim Sci 91:3930–3939

Duckworth DH, Gulig PA (2002) Bacteriophages: potential treatment for bacterial infections. BioDrugs 16:57–62

Espinoza-Ortega A, Espinosa-Ayala E, Bastida-López J, Castañeda-Martínez T, Arriaga-Jordán CM (2007) Small-Scale dairy farming in the highlands of central Mexico: technical, economic and social aspects and their impact on poverty. Exp Agric 43:241–256

Fadul-Pacheco L, Wattiaux MA, Espinoza-Ortega A, Sánchez-Vera E, Arriaga-Jordán CM (2013) Evaluation of sustainability of smallholder dairy production systems in the highlands of Mexico during the rainy season. Agroecol Sust Food Syst 37:882–901

García P, Madera C, Martínez B, Rodríguez A (2007) Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. Int Dairy J 17:1232–1239

García P, Martínez B, Obeso JM, Lavigne R, Lurz R, Rodríguez A (2009) Functional genomic analysis of two *Staphylococcus aureus* phages isolated from the dairy environment. Appl Environ Microbiol 75:7663–7673

Gill LL, Pacan JC, Carson ME, Leslie KE, Griffiths MW, Sabour PM (2006) Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. Antimicrob Agents Chemother 50:2912–2918

Gutiérrez D, Martínez B, Rodríguez A, García P (2010) Isolation and characterization of bacteriophages infecting *Staphylococcus epidermidis*. Curr Microbiol 61:601–608

Gutiérrez D, Martín-Platero AM, Rodríguez A, Martínez-Bueno M, García P, Martínez B (2011) Typing of bacteriophages by randomly amplified polymorphic DNA (RAPD-PCR) to assess genetic variability. FEMS Microbiol Lett 322:90–97

Gutiérrez D, Delgado S, Vázquez-Sánchez D, Martínez B, Cabo ML, Rodríguez A, Herrera JJ, García P (2012) Incidence of *Staphylococcus aureus* and analysis of associated bacterial communities on food industry surfaces. Appl Environ Microbiol 78:8547–8554

Hemme T (2007) FCN dairy report 2007, international farm comparison network. IFCN Dairy Research Center, Kiel

Iandolo JJ, Worrell V, Groicher KH, Qian Y, Tian R, Kenton S, Dorman A, Ji H, Lin S, Loh P, Qi S, Zhu H, Roe BA (2002) Comparative analysis of the genomes of the temperate bacteriophages phi11, phi12 and phi13 of *Staphylococcus aureus* 8325. Gene 289(1–2):109–118

Kaur G, Malik RK, Mishra SK, Singh TP, Bhardwaj A, Singroha G, Vij S, Kumar N (2011) Nisin and class IIa bacteriocin resistance among *Listeria* and other foodborne pathogens and spoilage bacteria. Microb Drug Resist 17:197–205

Klostermann K, Crispie F, Flynn J, Meaney WJ, Paul Ross R, Hill C (2010) Efficacy of a teat dip containing the bacteriocin lacticin 3147 to eliminate Gram-positive pathogens associated with bovine mastitis. J Dairy Res 77:231–238

Le Loir Y, Baron F, Gautier M (2003) *Staphylococcus aureus* and food poisoning. Genet Mol Res 2:63–76

Lee JH (2003) Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol 69:6489–6494

Lee SH, Camargo CH, Gonçalves JL, Cruz AG, Sartori BT, Machado MB, Oliveira CA (2012) Characterization of *Staphylococcus aureus* isolates in milk and the milking environment from small-scale dairy farms of São Paulo, Brazil, using pulsed-field gel electrophoresis. J Dairy Sci 95:7377–7383

Makovec JA, Ruegg PL (2003) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. J Dairy Sci 86:3466–3472

Manjarrez-López AM, Díaz-Zarco S, Salazar-García F, Valladares-Carranza B, Gutiérrez- Castillo C, Barbabosa-Pliego A, Talavera-Rojas M, Alonso-Fresan MU, Velázquez-Ordóñez V (2012) *Staphylococcus aureus* biotypes in cows presenting subclinical mastitis from family dairy herds in the Central-Eastern State of Mexico. Rev Mex Cien Pec 3(2):265–274

Martínez B, Bravo D, Rodríguez A (2005) Consequences of the development of nisin-resistant *Listeria monocytogenes* in fermented dairy products. J Food Prot 68:2383–2388

Notarnicola AM, Zamarchi GR, Onderdonk AB (1985) Misidentification of mucoid variants of *Staphylococcus aureus* by standard laboratory techniques. J Clin Microbiol 22:459–461

OIE (2014) Word Organisation of Animal Health. List of antimicrobial agents of veterinary importance. http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/OIE_list_antimicrobials.pdf. Accessed January 2014

Omoe K, Ishikawa M, Shimoda Y, Hu DL, Ueda S, Shinagawa K (2002) Detection of *seg*, *se*h, and *se*i genes in *Staphylococcus aure*us isolates and determination of the enterotoxin productivities of *S. aureus* isolates harbouring *seg*, *seh*, or *sei* genes. J Clin Microbiol 40(3):857–862

Pinchuk IV, Beswick EJ, Reyes VE (2010) Staphylococcal enterotoxins. Toxins (Basel) 2:2177–2197

Rall VLM, Miranda ES, Castilho IG, Camargo CH, Langoni H, Guimarães FF, Araújo Júnior JP, Fernandes Júnior A (2014) Diversity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis. J Dairy Sci 97:829–837

Sakwinska O, Giddey M, Moreillon M, Morisset D, Waldvogel A, Moreillon P (2011) *Staphylococcus aureus* host range and human-bovine host shift. Appl Environ Microbiol 77:5908–5915

Sánchez-Hidalgo M, Montalbán-López M, Cebrián R, Valdivia E, Martínez-Bueno M, Maqueda M (2011) AS-48 bacteriocin: close to perfection. Cell Mol Life Sci 68:2845–2857

Straub JA, Hertel C, Hammes WP (1999) A 23S rDNA-targeted polymerase chain reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. J Food Prot 62:1150–1156

Struelens MJ (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clin Microbiol Infect 2:2–11

Valle J, Latasa C, Gil C, Toledo-Arana A, Solano C, Penadés JR, Lasa I (2012) Bap, a biofilm matrix protein of *Staphylococcus aureus* prevents cellular internalization through binding to GP96 host receptor. PLoS Pathog 8:e1002843

Van Cleef BA et al (2011) Livestock associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis 17:502–505

Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS (2003) Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 92(1–2):179–185

Waldvogel FA (2001) *Staphylococcus aureus* (including staphylococcal toxic shock). In: Mandell JL, Bennett JE, Dolin R (eds) Principles and practice of infection diseases. Churchill Livingstone, Philadelphia, PA

Wang SC, Wu CM, Xia SC, Qi YH, Xia LN, Shen JZ (2009) Distribution of superantigenic toxin genes in *Staphylococcus aureus* isolates from milk samples of bovine subclinical mastitis cases in two major diary production regions of China. Vet Microbiol 137:276–281

Wilson IG, Cooper JE, Gilmour A (1991) Detection of enterotoxigenic *Staphylococcus aureus* in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes entB and entC1 and the thermonuclease gene *nuc*. Appl Environ Microbiol 57:1793–1798