Microbiological Research 168 (2013) 77–83

Contents lists available at SciVerse ScienceDirect

Microbiological Research

journal homepage: www.elsevier.de/micres

Genotypic diversity and virulent factors of *Staphylococcus epidermidis* isolated from human breast milk

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ARTICLE INFO

Article history:
Received 7 May 2012
Received in revised form 8 August 2012
Accepted 13 September 2012

Keywords:
Human breast milk
S. epidermidis
Antibiotic susceptibility
Virulence

ABSTRACT

Staphylococcus epidermidis strains were isolated from the expressed human breast milk (EHM) of 14 healthy donor mothers. Genetic diversity was evaluated using RAPD-PCR REP-PCR and pulse-field gel electrophoresis (PFGE). PFGE allowed the best discrimination of the isolates, since it provided for the greatest diversity of the analyzed genomes. Among the *S. epidermidis* strains, resistance to gentamicin, tetracycline, erythromycin, clindamycin or vancomycin was detected, whilst four isolates were multiresistant. The results from our study demonstrate that staphylococci from EHM could be reservoirs of resistance genes, since we showed that *tetK* could be transferred from EHM staphylococci to Gram-negative *Escherichia coli*. Most of the staphylococcal strains displayed excellent proteolytic and lipolytic activities. Additionally, the presence of *ica* genes, which was related to their ability to form a biofilm on tissue culture plates, and the presence of virulence factors including autolysin/adhesin AtLE, point to their pathogenic potential.

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1. Introduction

The development of gut microbiota starts at birth and is influenced by feeding and the environment (Penders et al. 2006). Expressed human breast milk (EHM) represents an important source of potentially beneficial bacteria to the infant's gut, including lactic acid bacteria (LAB), staphylococci and streptococci (Jiménez et al. 2008; Perez et al. 2007). *Staphylococcus epidermidis* appears to be the predominant bacteria in human milk (Martín et al. 2007). However, this species has received marginal attention regarding its contribution to and role in the process of early colonization of the infant's gut. Bacteria residing in the human gut are involved in processes important to both health and disease (Eckburg et al. 2005). One of the features of *S. epidermidis* is its widespread colonization of the human epithelia, where it conducts commensal and potentially infectious lifestyles (Otto 2009). Also, it is believed to be more readily transmissible than other representatives of the genus, including the virulent species *Staphylococcus aureus* (Massey et al. 2006). As part of the human epithelial microflora, *S. epidermidis* is thought to have a benign relationship with the host. Nevertheless, among coagulase-negative staphylococci, *S. epidermidis* is responsible for the greatest number of infections, although usually without severe clinical manifestations (Otto 2009).

This species is found predominantly in the lipid-rich regions of the human organism (skin or EHM), and like most organisms colonizing such areas, it possesses a degree of lipolytic activity (Longshaw et al. 2000). Lipases may be important for the colonization and persistence of *S. epidermidis* in the gut and they most likely have an indirect influence on their potential, either beneficial or pathogenic, capacity (Longshaw et al. 2000). Nevertheless, for *S. aureus*, lipases are considered as virulence factors, along with extracellular proteases and adhesive proteins (Saxena and Gomber 2010). Moreover, it has been demonstrated that staphylococci possess different extracellular proteinases related to their virulence, but only a few studies of *S. epidermidis* extracellular proteinases have been conducted (Kawano et al. 2001; Saxena and Gomber 2010).

The ability of *S. epidermidis* to form a biofilm is apparently linked with the infections caused by this species. Among other factors, the intracellular adhesin encoded by the *ica* locus and AtLE autolysin/adhesin (a vitronectin-binding cell surface protein) play significant roles in its formation (Sivadon et al. 2009). These genes are virulence markers and their presence in the *S. epidermidis* species is considered an indicator of the pathogenic potential of the strain (Aricola et al. 2001).

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0944-5013/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.micres.2012.09.004

Moreover, the rate of antibiotic resistance of staphylococci has increased in the past several years (Casey et al. 2007). Previous results have demonstrated that EHM could be a reservoir of multiresistant staphylococci (Carneiro et al. 2004). Due to continuous contact and interactions with other bacteria in the human gut, the exchange of the genes responsible for the antibiotic resistance occurs between them (Levy 2002).

The aims of the present study were: (a) to provide information on the genotypic diversity of *S. epidermidis* from human breast milk and to find the most discriminative method for genotyping; (b) to evaluate its virulence potential through the assessment of proteolytic activity, lipolytic activity and the occurrence of genes related to biofilm formation; and (c) to define the antibiotic susceptibility patterns of the studied organisms and disseminate the potential of antibiotic resistance determinants.

2. Materials and methods

2.1. Bacterial strains and cultivation

The strains used in this study are part of a laboratory collection created in 2009 from 14 different donors with no clinical symptoms of mastitis (from 3rd to 5th day of lactation). The breasts were cleaned with antiseptic soap and rinsed with sterile water. After the rejection of 2–3 ml of foremilk, EHM was manually expressed in sterile plastic tubes using sterile gloves. As a control, a swab of the areola was taken before milk collection. The samples were transported in a cold box (+4 °C) and immediately processed upon arrival in the laboratory. *S. epidermidis* isolates were grown in Brain Heart Infusion medium (BHI) (Merck, Darmstadt, Germany) at 37 °C. Overall, 140 isolates (10/woman), Gram-positive, catalase-positive, lysozyme-resistant and microscopically confirmed as cocci, were selected. Based on preliminary tests and BOX PCR, the isolates from each individual were uniform and were regarded as different isolates of the same strain. This is why only one representative sample per woman was chosen for further study.

2.2. Molecular identification and fingerprinting

Total genomic DNA was prepared according to the method described by Hopwood and collaborators (1985). PCR with primers that encompassed 1492 bp of the 16S rDNA (UNI16SF 5′-GAG AGT TTG ATC CTG GC-3′ and UNI16SR 5′-AGG AGG TGA TCC AGC CG-3′) were used in PCR reactions according to Jovcic and co-authors (2009). The PCR products were purified by the QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and subjected to sequencing by the MACROGEN service (Macrogen Inc., Seoul, Korea).

RAPD-PCR (with RAPD1 or M13R2 primers) and REP-PCR (with $(GTG)_5$, ERIC or BOX primers) were performed as previously described (Kersulyte et al. 1992; De la Puente-Redondo et al. 2000; Versalovic et al. 1994). PCR amplicons were separated on 1.5% agarose gels, visualized on a UV transilluminator and photographed.

PFGE profiles were obtained using a contour-clamped homogeneous electric field system (2015-131 Pulsafor unit, LKB-Pharmacia, Sweden) in 0.5 × TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA), as described previously (Kojic et al. 2006). For electrophoretically resolving *Sma*I restriction fragments, the pulse times were linearly increased from 8 to 18 s during 18 h at 300 V. Lambda Ladder PFG Marker (Biolabs, UK) served for size determination. Clustering was done using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

2.3. Antimicrobial susceptibility and detection of antibiotic resistance genes

The MICs for ampicillin, erythromycin, clindamycin, tetracycline, gentamicin and vancomycin were determined using the broth microdilution test (Clinical and Laboratory Standard Institute) (CLSI 2011). *S. aureus* ATCC25923 was used as a quality control.

The presence/absence of the genes involved in antibiotic resistance in staphylococci was determined using PCR with the appropriate primers (Table 1).

2.4. Transformation of *E. coli* DH5α

For plasmid isolation, a JETSTAR Plasmid Purification Mini Kit was used (Genomed, Löhne, Germany). Upon isolation, competent antibiotic-sensitive *E. coli* DH5α cells were transformed with plasmid DNA and transformants were selected for resistance on Luria Broth agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar in water) and containing the respective antibiotic (Hanahan 1983).

2.5. Assays of proteolytic activity

Detection of caseinolytic activity was performed as previously described (Kojic et al. 1991). α,β,κ-Casein solutions (0.5%) were prepared in 100 mM sodium phosphate buffer (pH 6.8) (Sigma–Aldrich Chemie GmbH, Germany). The degradation of bovine milk casein fractions was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described (Kojic et al. 1991).

2.6. Assay of lipolytic activity

For detection of the lipolytic activity of *S. epidermidis*, the strains were streaked onto tributyrin agar plates (Torlak, Serbia) and incubated for 24 h at 37 °C as proposed by Anderson (1939). The degradation of tributyrin from the culture medium gives rise to clear zones surrounding the lipolytic colonies.

2.7. Detection of biofilm formation

In the present study, we screened all the isolates for their ability to form a biofilm by the tissue culture plate method (TCP) as described by Christensen and co-authors (1985); this test is considered a standard test for the detection of biofilm formation. Wells that contained only BHI medium served as a negative control.

Additionally, the presence of genes related to biofilm formation on (*ica*) was assessed by PCR amplification. Primers and annealing temperatures for the amplification of *icaA*, *icaB*, *icaC* and *atlE* genes are listed in Table 1. In addition, the presence of the virulence factor autolysin/adhesin AtLE that is also related to the attachment of bacteria to different surfaces, was checked by PCR using primers specific for this gene (Table 1). PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.

2.8. Sequencing of PCR products

All PCR products were purified (QIAquick PCR Purification Kit, QIAGEN GmbH, Germany) and sequenced with the appropriate forward primers. Sequencing was performed by the MACROGEN service (Macrogen Inc., Seoul, Korea) and sequences were analyzed using BLAST (www.ncbi.nlm.nih.gov).

Table 1
Primers used in the study.

Primer name	Nucleotide sequence (5′ → 3′)	Target gene (function)	Ta (°C)[a]	PCR product size (bp)	Reference
TETK-FW1 TETK-RV1	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAAACTCTTGAAA	*tetK* (efflux pump)	55	382	Gevers et al. (2003)
TETL-FW3 TETL-RV3	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCCACCTAA	tetL (efflux pump)	55	717	Gevers et al. (2003)
Ribo2-FW Ribo2-Rv	GGMCAYRTGGATTTYWTIGC TCIGMIGGIGTRCTIRCIGGRC	RPP[b]	"Touchdown"	1187	Aminov et al. (2001)
ERMA-FW ERMA-RV	AAGCGGTAAACCCCTCTGA TTCCCAAATCCCTTCTCAAC	*ermA* (methylase)	55	190	Strommenger et al. (2003)
ErmB-Fw ErmB-Rev	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	*ermB* (methylase)	55	425	Jensen et al. (1999)
ErmC-Fw ErmC-Rev	ATCTTTGAAATCGGCTCAGG CAAACCCGTATTCCACGATT	*ermC* (methylase)	55	295	Jensen et al. (1999)
Gen1 Gen2	ATATGATTATGAAAAAGGTGA ATAATCAATCTTTATAAGTCC	*aac(6′)-Ie* (ACT)[c]	50	1470	Rouch et al. (1987)
VANA VANA1	TGAATAGAATAAAAGTTGCAATAC CCCCTTTAACGCTAATACGAT	*vanA* (ligase)	62	1029	Dutka-Malen et al. (1990)
VANB VANB1	CCCGAATTTCAAATGATTGAAAA CGCCATCCTCCTGCAAAA	*vanB* (ligase)	59	457	Evers et al. (1994)
VANC VANC1	GCTGAAATATGAAGTAATGACCA CGGCATGGTGTTGATTTCGTT	*vanC* (ligase)	58	811	Dutka-Malen et al. (1992)
icaA-F icaA-R	GACCTCGAAGTCAATAGAGGT CCCAGTATAACGTTGGATACC	*icaA* (ligase)	50	814	Ziebuhr et al. (1999)
icaB-F icaB-R	ATGGCTTAAAGCACACGACGC TATCGGCATCTGGTGTGACAG	*icaB* (ligase)	56	526	Ziebuhr et al. (1999)
icaC-F icaC-R	ATAAACTTGAATTAGTGTATT ATATATAAAACTCTCTTAACA	*icaC* (ligase)	42	989	Ziebuhr et al. (1999)
atlE-F atlE-R	CAACTGCTCAACCGAGAACA TTTGTAGATGTTGTGCCCCA	*atlE* (ligase)	50	682	Frebourg et al. (2000)

[a] Annealing temperature.
[b] Ribosomal protection proteins.
[c] Aminoglycoside acetyltransferase.

3. Results

3.1. Molecular identification

The preliminary species determination of the 14 *S. epidermidis* isolates was confirmed by 16S rDNA sequencing; nucleotide sequences were submitted to EMBL Nucleotide Sequence Database under accession numbers: [BGHMN1 – EMBL: FR797794], [BGHMN2 – EMBL: FR797795], [BGHMN3 – EMBL: FR797796], [BGHMN4 – EMBL: FR797797], [BGHMN5 – EMBL: FR797798], [BGHMN7 – EMBL: FR797799], [BGHMN8 – EMBL: FR797800], [BGHMN9 – EMBL: FR797801], [BGHMN10 – EMBL: FR797802], [BGHMC1 – EMBL: FR797803], [BGHMC5 – EMBL: FR797804], [BGHMC8 – EMBL: FR797805], [BGHMC9 – EMBL: FR797806], [BGHMC11 – EMBL: FR797807].

3.2. Molecular fingerprinting

The fingerprints of the *S. epidermidis* strains were obtained with five different primers (RAPD1, M13R2, $(GTG)_5$, ERIC and BOX) and the sorting of the *S. epidermidis* strains according to UPGMA into different fingerprint groups is presented in Table 2. REP-PCR amplification with BOX and ERIC primers enabled the discrimination of five pattern groups, each containing 3–10 DNA fragments between 300 and 1500 bp, respectively (Table 2). The fingerprints obtained with $(GTG)_5$-PCR contained up to 15 DNA fragments between 800 bp and 3 kb and were separated into six fingerprint groups (Table 2). RAPD-PCR yielded four distinct fingerprints when RAPD1 primers were used for strain typing, and eight fingerprints when RAPDM13 primers were used (Table 2). RAPD1-PCR gave the band pattern containing the lowest number of DNA fragments (2–6) with a size range between 900 bp and 2 kbp. When M13 was used, all *S. epidermidis* fingerprints shared distinct common DNA fragments present at 450 and 650 bp; the number of fragments was higher than in RAPD1-PCR (5–11), with sizes between 200 and 1500 bp.

For a more detailed examination of the genetic diversity of *S. epidermidis*, the isolates were subjected to PFGE genotyping using the *Sma*I restriction enzyme (Fig. 1). Analysis of the fingerprints revealed 11 different pulsotypes among the isolates. Isolates BGHMN2 and BGHMN3, BGHMN9 and BGHMC11, BGHMC1 and BGHMC5, belonged to three different fingerprint types, respectively, as determined by UPGMA.

3.3. Proteolytic activity of S. epidermidis

The proteolytic activities of the strains were classified into four categories based on the α, β and κ casein fraction degradation profiles on SDS gels: high, medium, low or no degradation. Nine of the fourteen strains showed medium to high proteolytical activity and degraded all three casein fractions (not shown). The *S. epidermidis* strains were more efficient in β-casein and κ-casein degradation (most exhibited medium to high activity) than in α-casein degradation. Amongst the analyzed isolates, strain BGHMC9 was the most efficient since it completely hydrolyzed all three casein fractions (not shown). Two strains, BGHMN7 and BGHMC1, showed the lowest potential to degrade major casein fractions.

3.4. Lipolytic activity of S. epidermidis

According to the test on tributyrin agar, all 14 strains exhibited excellent lipolytic activity, producing clear zones around the bacterial growth approximately 10 mm in width (not shown).

3.5. Antimicrobial susceptibility

The distribution of MIC values for the six antibiotics obtained in the microdilution test is summarized in Table 3. All strains were susceptible to ampicillin (MIC ≤ 8 mg/L) and resistant to vancomycin (MIC = 8 mg/L). It should be noted that MICs obtained for vancomycin indicate intermediate resistance to this antibiotic.

Table 2
Classification of *S. epidermidis* strains into groups based on fingerprints obtained in REP-PCR (BOX, ERIC and $(GTG)_5$ primers) and RAPD-PCR (RAPD1 and RAPDM13).

Fingerprint group	Primer used for fingerprinting				
	BOX	ERIC	$(GTG)_5$	RAPD1	RAPDM13
I	BGHMN1 BGHMN2 BGHMN4 BGHMN5 BGHMN7 BGHMN10 BGHMC1 BGHMC5	BGHMN1 BGHMN2 BGHMN3 BGHMN5 BGHMC8 BGHMC9 BGHMC11	BGHMN1 BGHMC8 BGHMC11	BGHMN1 BGHMN4 BGHMN9 BGHMN10 BGHMC8	BGHMN1
II	BGHMN3	BGHMN4	BGHMN2 BGHMN3 BGHMC9	BGHMN2 BGHMN3 BGHMN5 BGHMN7 BGHMC1 BGHMC9 BGHMC11	BGHMN2
III	BGHMN8 BGHMN9 BGHMC9	BGHMN7 BGHMN10 BGHMC1 BGHMC5	BGHMN4 BGHMC1	BGHMC5	BGHMN3
IV	BGHMC8	BGHMN8	BGHMN5 BGHMN7 BGHMN8	BGHMN8	BGHMN4 BGHMN5 BGHMN8 BGHMN9 BGHMC1
V	BGHMC11	BGHMN9	BGHMN9		BGHMN7
VI			BGHMN10 BGHMC5		BGHMN10 BGHMC11
VII					BGHMC5 BGHMC8
VIII					BGHMC9

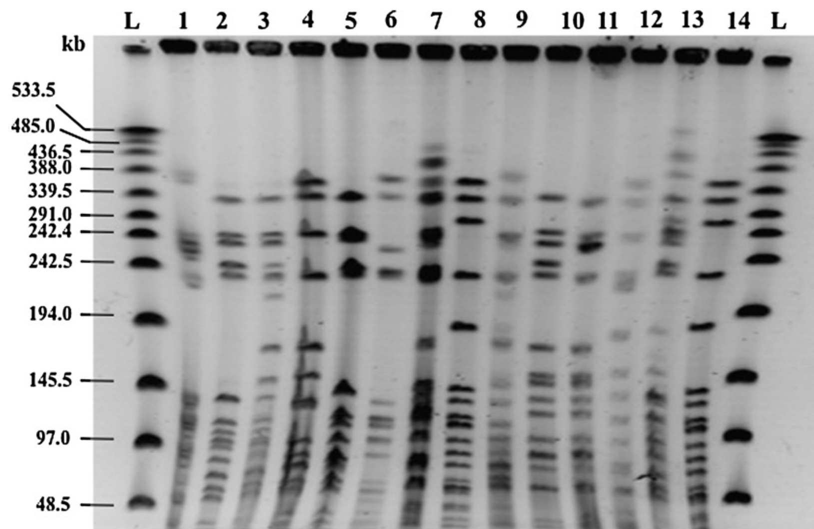

Fig. 1. Pulse-field gel electrophoresis patterns of *Sma*I-digested genomic DNA of *S. epidermidis* strains from human breast milk. Lanes L–λ concatemers: 1. BGHMN1, 2. BGHMN2, 3. BGHMN3, 4. BGHMN4, 5. BGHMN5, 6. BGHMN7, 7. BGHMN8, 8. BGHMN9, 9. BGHMN10, 10. BGHMC1, 11. BGHMC5, 12. BGHMC8, 13. BGHMC9, 14. BGHMC11.

Table 3
Distribution of MICs to six antibiotics in *S. epidermidis* strains isolated from human breast milk.

Antibiotic	MIC (g/L)											
	≤0.125	0.25	0.5	1	2	4	8	16	32	64	*n*	BV
Ampicillin	14										14	0.25
Erythromycinn		7	5						2		14	8
Clindamycin	10	1	1						2		14	8
Tetracycline				5		3	1			5	14	16
Gentamicin			7						7		14	8
Vacomycin							14				14	2

n – number of tested isolates; BV – MIC breakpoint value according to CLSI (2011).

A significant number of strains exhibited resistance to gentamicin (seven strains) with MICs of 32 mg/L, tetracycline (five strains) (MIC = 64 mg/L), and two strains were resistant to erythromycin and clindamycin (MIC = 32 mg/L). Four *S. epidermidis* strains, BGHMN4 (Tet^r, Ery^r, Cln^r, Van^r), BGHMN10 (Tet^r, Ery^r, Cln^r, Van^r), BGHMC5 (Tet^r, Gen^r, Van^r) and BGHMC11 (Tet^r, Gen^r, Van^r), were multiresistant. It should be noted that MICs obtained for vancomycin (≤8 mg/L) indicate intermediate resistance to this antibiotic.

Although BGHMN9 and BGHMC11 belonged to the same pulsotype, they exhibited different antibiotic susceptibility patterns.

For tetracycline-resistant *S. epidermidis*, PCRs with different primers specific for the genes that confer resistance to this antibiotic were performed (Table 1). When *tetK* specific primers were used in reactions, a DNA fragment of 382 bp was obtained for all five tetracycline-resistant strains. Sequencing of the obtained fragments and BLAST-assisted alignment of the nucleotide sequences showed 97–98% identity with the tetracycline efflux protein (*tetK*) from *S. aureus* plasmid pT181. The *ermB* gene was detected for two erythromycin-resistant strains and according to BLAST analysis, both PCR fragments shared 98% identity with the *ermB* genes from different streptococci. Additionally, the sequencing of the 1470 bp fragment that was obtained with primers for aminoglycoside acetyltransferase (*aac(6′)-Ie* gene) in all gentamicin-resistant strains, revealed 98% identity with 6′-amino-N-acetyltransferase from *S. aureus* by BLAST analysis. For the vancomycin-resistant strains, products were not detected when primers specific for *vanA*, *vanB* and *vanC* genes were used in the reactions.

3.6. Potential of antibiotic resistance gene dissemination

Five *S. epidermidis* strains resistant to tetracycline (Tet^r) (BGHMN4, BGHMN10, BGHMC1, BGHMC5 and BGHMC11) served as donors of plasmids in the transformation-mediated transfer of tetracycline resistance to tetracycline sensitive *E. coli* DH5α (Tet^s). Plasmids isolated from donor strains were transferred into the donor strain through transformation. After transformation, between 20 and 30 *E. coli* tetracycline-resistant colonies were detected for each strain that served as a plasmid donor. The results of PCR analysis confirmed the presence of the *tetK* gene in all transformants. Nevertheless, we could not demonstrate in the same way the horizontal transfer of *aac(6′)-Ie* (from BGHMC11), *ermB* (from BGHMN10), nor resistance to vancomycin (from BGHMN10). Moreover, the transformation of *E. coli* with plasmids from the BGHMN4 or BGHMN10 strains (both Tet^r, Ery^r and Van^r), resulted in only *E. coli* (T^r) transformants, and these results were also confirmed with PCR.

3.7. Biofilm formation

In the TCP method (tissue culture plate method), out of the 14 isolates tested for biofilm formation, 12 isolates formed a strong biofilm on polystyrene microtiter plates. At the same time, two isolates (BGHMN9 and BGHMC1) were considered as non- or weak biofilm producers according to the TCP method (not shown). The presence of virulence genes was assessed *via* PCR with specific primers for genes in the *ica* operon and the *atlE* gene. Among the 14 isolates, 12 were positive for the presence of the *ica* operon, with PCR products obtained for the *icaA*, *icaB* and *icaD* genes. Only for BGHMN9 and BGHMC1 strains a specific PCR product was not obtained. Additionally, all isolates (14) were positive for the presence of the gene for AtlE autolysin.

4. Discussion

EHM represents a valuable source of different bacterial species for the infant's gut, including commensal, mutualistic, and/or probiotic bacteria (Martín et al. 2009). The complex gut microbiota is involved in different aspects of human health, and understanding the role of each inhabitant of this ecological niche is of great importance. These bacteria may play an important role in the preservation of the good health of a breast-fed infant, but it can also present a source of bacteria with potentially pathogenic features. Although immunocompromised patients are often at risk of developing more serious infections, it is believed that *S. epidermidis* could be regarded as an "accidental pathogen" (Otto 2009).

In this study, we have examined different aspects of *S. epidermidis* isolated from EHM in order to contribute to a better understanding of the potential pathogenicity that this species might have on gut colonization of breast-fed infants. Regardless of all the precautions and controls undertaken during the milk sampling, we could not entirely exclude the possibility that these strains originated from the mothers' skin (although no *S. epidermidis* was detected on the control plates). Nevertheless, our focus was to explore the pathogenic potential of *S. epidermidis* in the infant's gut, even if the source of this species was the skin.

Due to the worldwide presence and dissemination of *S. epidermidis*, it is necessary to determine the most advantageous molecular technique(s) to be used for the precise identification and differentiation of this species. Several studies have described the application of REP-PCR and RAPD PCR for the identification and differentiation of staphylococci (Wieser and Busse 2000). The authors demonstrated that ERIC- and BOX-PCR were excellent tools for the rapid identification of *S. epidermidis* strains at the species level. Still, in these analyses, *S. epidermidis* isolates from different sources showed low fingerprint diversity, particularly when BOX-PCR was performed. The results obtained from REP-PCR and RAPD-PCR analysis in our study revealed different strain types among the tested *S. epidermidis* strains. However, the primers used in the study were not equally successful in the differentiation of the strains. Based on our results, the RAPD-PCR fingerprints obtained with the RAPDM13 primer appeared to be the most informative for the tested strains. Nevertheless, PFGE results revealed a significantly greater diversity of the analyzed genomes. This is why we propose that, where possible, PFGE should be applied for the best differentiation among *S. epidermidis* strains.

EHM is generally accepted to be the best nutrition for neonates. It contains the necessary nutrients and antibacterial factors for the protection of the infant against infections (May 1994). Only several studies on the proteolytic activity of staphylococci were conducted, and this feature was related to the potential pathogenicity of this species (May 1994). The *S. epidermidis* strains from our study originated from EHM where they might be involved in protein degradation. A principal protein of human breast milk is casein, which is homologous to bovine β-casein (Jenness 1979). Most of the strains analyzed in our study degraded the β-casein fraction, pointing to the potential of *S. epidermidis* to degrade human milk proteins, thereby leading to the release of peptide fragments and amino acids (Smid et al. 1991). The proteolytic activity of the strains implies the presence of active staphylococcal proteinase(s) that were previously related to the process of abrogation of epidermal permeability barriers and proteolysis of tissue proteins such as collagen, myoglobin and fibrin (Hirasawa et al. 2010; Saxena and Gomber 2010).

The lipases found in human milk break down fat into small globules, rendering it easier to be digested and absorbed by an infant's gut. However, staphylococcal lipases along with other proteins also help the bacteria to degrade host components, thereby also assuming a role in bacterial virulence (Saxena and Gomber 2010).

Overall, the good proteolytic and lipolytic activities of the *S. epidermidis* isolated from EHM in this study could be related to the adaptation of this species to the specific environment they inhabit, indicating the virulent potential of these microorganisms.

Apart from some features of *S. epidermidis* that could be beneficial, we also observed that most of the strains carry genes associated with adherence to polymer surfaces and biofilm formation. All the isolates where the presence of the *ica* operon was detected were shown to form a strong biofilm on polystyrene microtiter plates. Infections caused by *S. epidermidis* are often associated with the use of medical devices and most commonly occur in immunocompromised patients (Ziebuhr et al. 1999). Epidemiological and experimental data strongly indicate that biofilm production contributes to the virulence properties of specific *S. epidermidis* strains (Ziebuhr et al. 1999). Molecular studies on biofilm formation suggest that the primary attachment of *S. epidermidis* to different surfaces is mediated by the genes encompassed in the *ica* operon. Moreover, autolysin AtlE also has adhesive properties and is suspected to play a role in the interaction between staphylococcal cells and biomaterial (Sivadon et al. 2009). The presence of genes associated with adhesion and biofilm formation in *S. epidermidis* has been linked to catheter and medical device-related sepsis (Kloos and Bannerman 1994). Nevertheless, two staphylococcal strains that lacked the *ica* operon and were only positive for the presence of *atlE* did not form a biofilm on a polypropylene surface, at least none that could be detectable by this procedure. This might indicate that this gene may contribute to biofilm formation to a lesser extent and that the *ica* operon is responsible for biofilm formation on this type of material. In addition, since *S. epidermidis* is the most common cause of nosocomial infections in neonates (De Silva et al. 2002), the results from our study reveal the pathogenic potential of isolated *S. epidermidis.*

Another potentially undesirable characteristic of the bacterial strain that colonizes the human gut is the potential to disseminate antibiotic resistance genes. The excessive use of antibiotics has intensified the emergence and dissemination of antibiotic resistance genes among bacteria (Levy 2002). Transformation is one of mechanisms of gene transfer, where competent bacteria obtain and maintain genetic variability by the "sampling" of DNA-like plasmids from their surroundings and acquire new adaptive traits, such as resistance to antibiotics (Nielsen et al. 2000; Sørensen et al. 2005). EHM presents a primary source of bacteria for the infant's gut, and the role of transformation in the antibiotic resistance dissemination of *S. epidermidis* was investigated in our study. Resistance to gentamicin, tetracycline, erythromycin, clindamycin and vancomycin was observed and partially linked to the respective resistance-conferring genes. Nevertheless, for the strains showing an intermediate resistance to vancomycin, the presence of *vanA*, *vanB* or *vanC* genes was not detected. This mechanism of intermediate resistance to vancomycin has not been associated with the resistance genes and is still the subject of intensive research (Cui et al. 2006). One of the proposed mechanisms is the cooperative effect of the clogging and cell wall thickening that prevents vancomycin from reaching the cell membrane and inhibiting cell wall growth (Cui et al. 2006).

We have also explored the potential of the transformation-mediated transfer of detected resistance from different *S. epidermidis* strains to the *E. coli* DH5α sensitive strain. According to our results, the tetracycline resistance *tetK* gene is located on the plasmid in all tetracycline-resistant *S. epidermidis*, and it could be horizontally transferred to another species such as *E. coli*. Alternatively, *ermB*, *van* and *aac(6′)-Ie* genes might be located either on the bacterial chromosome or on a another plasmid that is difficult to isolate or transfer to another bacteria. Nonetheless, we have demonstrated the transformation-mediated horizontal transfer of *tetK* and the implicated potential threat of further horizontal transfer of resistance determinants in an infant's gut. This calls for more detailed analyses of antibiotic resistance among human microbiota.

The bacteria present in EHM have a major effect on the microbial composition in the infant gut. Therefore, *S. epidermidis* that is often present in EHM most likely has a different impact on infant's health during the early colonization of the gut. Although the proteolytic and lipolytic activities of *S. epidermidis* might be beneficial features of this species, they also present potential virulence factors. Moreover, the potential of antibiotic resistance dissemination between species represents a great problem for the transfer of the resistance genes in complex bacterial communities as found in the human gut. Our study has revealed that *S. epidermidis* isolated from EHM could be a vehicle for the dissemination of antibiotic resistance. Additionally, the results on the biofilm formation potential and the presence of virulence factors strongly indicate that staphylococci isolated from EHM may present a health hazard since they can be regarded as potential pathogens. Taking into account all the data from our study, it is necessary to perform more detailed studies of the roles that can be assumed by the different bacterial species in the human gut, particularly during the phase of early colonization. These studies should include not only "beneficial" species but other species, like *S. epidermidis* that are often defined to be somewhere between a commensal and pathogenic, and which potentially pose a risk for the newborns.

Acknowledgments

This work was supported by the Ministry of Education and Science of the Republic of Serbia, Grant No. 173019. We are grateful to Ms. Myra Macpherson Poznanović, official native English editor of the scientific journal Archives of Biological Sciences.

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