Original paper

Activity of antimicrobial peptides and conventional antibiotics against superantigen positive *Staphylococcus aureus* isolated from patients with atopic dermatitis

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Abstract

Introduction: *Staphylococcus aureus* causes a diverse array of diseases, ranging from relatively harmless localized skin infections to life-threatening systemic conditions. It secretes toxins directly associated with particular disease symptoms.
Aim: To determine the prevalence of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) colonization among patients with atopic dermatitis and to assess the antimicrobial susceptibility to conventional antibiotics and selected antimicrobial peptides among toxin-producing strains and nonproducing strains.
Material and methods: One hundred patients with atopic dermatitis and 50 healthy people were microbiologically assessed for the carriage of *S. aureus*. Antimicrobial susceptibility tests were performed using the broth microdilution method for conventional antibiotics and antimicrobial peptides (CAMEL, Citropin 1.1, LL-37, Temporin A). Detection of genes *lukS/lukF-PV*, *tst*, *sea-sed*, *eta* and *etb* by multiplex PCR was performed.
Results: *Staphylococcus aureus* strains were isolated from the majority of patients, from either the skin (75%) or the anterior nares (73%). Among the conventional antibiotics tested, the highest rates of resistance were observed for ampicillin, daptomycin, lincomycin and erythromycin. Antimicrobial peptides did not show significant diversity in activity. Among MSSA strains greater differentiation of secreted toxins was observed (*sec*, *eta*, *pvl*, *tsst*, *etb*, *seb*), while in the group of MRSA strains secretion of 3 toxins (*pvl*, *eta*, *seb*) was noted.
Conclusions: Antimicrobial resistance continues to evolve. It is important to monitor *S. aureus* infections. The profile of toxins produced by *S. aureus* strains is an important consideration in the selection of an antimicrobial agent to treat infections.

Key words: antimicrobial peptides, atopic dermatitis, polymerase chain reaction.

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease with a chronic course. Patients suffering from AD show increased susceptibility to infections caused by viruses, bacteria or fungi and have an altered skin microflora. Studies have shown that 80 to 100% of patients with AD present nasal or skin colonization by *Staphylococcus aureus*, while the prevalence is 5 to 30% in healthy individuals [1]. A correlation between the severity of the eczema and colonization with *S. aureus* has been demonstrated, and it has been determined that bacterial colonization is an important factor aggravating skin lesions [2]. The pathogenicity of *S. aureus* is associated with the production of staphylococcal superantigens, which include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEA, SEB, SEC, SED), exfoliative toxins (ETA and ETB), and leukocidin [3]. Superantigens are characterized by their capacity to stimulate a large number of T-cells. In contrast to conventional antigens, superantigens avoid intracellular processing and bind directly to the major histocompatibility complex (MHC) class II molecule, on the surface of the antigen processing cell, outside the antigen-binding groove [4]. Recently, an epidemic of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infections has emerged throughout the United States. These

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strains have an SCC*mec* (staphylococcal cassette chromosome mec) type IV cassette conferring resistance to methicillin, and 77% of them harbor genes for Panton Valentine leukocidin (PVL) [5, 6]. In addition, the prevalence of the genes for α-toxin and SEB is higher in CA-MRSA than in hospital-acquired MRSA (HA-MRSA), suggesting that strains circulating in the community are more virulent than hospital-associated strains [7]. The enhanced resistance of bacteria to conventional antibiotics is a serious problem in present day healthcare; thus the development of novel antimicrobial therapies, such as those based on various antimicrobial peptides (AMPs), seems to be advisable. The human body is equipped with more than 100 antimicrobial peptides that are an integral part of innate immunity [8]. They have broad antibacterial activity against Gram-positive and -negative bacteria and also show antifungal and antiviral activity. AMPs kill bacteria by permeating their membranes, and thus the lack of a specific molecular microbial target minimizes resistance development [9]. Currently, several peptides and peptide-based compounds are undergoing clinical trials [10]. Lesional skin of AD patients shows less expression of AMPs than would be predicted based on the inflammation and skin damage at this site [9]. Decreased expression of human β defensins (hBD-2 and hBD-3) in AD was also observed, and Th-2 cytokines IL-4 and IL-13 suppress hBD-2 and hBD-3 mRNA induction by tumor necrosis factor α (TNF-α) in keratinocytes [11]. Decreased expression of AMPs can explain the increased susceptibility of AD patients to skin infection.

Aim

The main purpose of this study was to assess the colonization of *S. aureus* in patients with AD. The isolated bacterial strains were analyzed for superantigen excretion and susceptibility to conventional antibiotics and selected AMPs (CAMEL, Citropin 1.1, LL-37, and Temporin A).

Material and methods

Patients and bacterial isolates

Patients were enrolled in our study during their visits in the Outpatient Clinic and hospitalization in the Department of Dermatology, Venereology and Allergology in Gdańsk (Medical University in Gdańsk) from August 2014 to August 2015. There was no selection of patients by sex or by severity of lesions. Atopic dermatitis was diagnosed following the criteria of Hanifin and Rajka, which include: pruritus, typical morphology and distribution of eczematous lesions, chronicity of the disease and personal or family history of atopy [12]. The study was approved by the local Research Ethics Board (approval number NKBBN/242-477/2014). Voluntary informed consent in written form was obtained from all participants. The exclusion criteria included: chronic dermatological condition with compromised skin barrier (e.g. psoriasis), diagnosis of any other chronic condition that increases the risk for MRSA colonization, oral or intravenous antibiotic treatment in the previous 4 weeks, treatment with topical antibiotics in the past 2 weeks, treatment with systemic corticosteroids or immunosuppressive drugs in the past 4 weeks, history of hospitalization, surgery, dialysis or residence in a long-term facility in the past year, indwelling catheter or a percutaneous device at the time of enrollment. Skin and nasal swabs collected from 100 patients with AD and 50 controls were used to investigate the presence of *S. aureus*. The control group consisted of patients without personal or family history of skin or allergic diseases who visited the Dermatological Outpatient Clinic for the dermatoscopic evaluation of moles. As a matter of fact, the CA-MRSA definition introduced in 2000 year by the Centers for Disease Control and Prevention (CDC) is described as a MRSA infection in a person who has none of the following established risk factors for MRSA infection: isolation of MRSA more than 48 h after hospital admission; history of hospitalization, surgery, dialysis or residence in a long-term care facility within one year of the MRSA culture date; the presence of an indwelling catheter or a percutaneous device at the time of culture; or previous isolation of MRSA [13].

Identification of *S. aureus* and MRSA strains

Preliminary identification and detection of *S. aureus* and MRSA strains was conducted using ChromID MRSA/ChromID *S. aureus* biplate (bioMérieux) for the simultaneous detection of *S. aureus* and methicillin-resistant *S. aureus* (MRSA).

Antimicrobial agents

Antimicrobial susceptibility tests for conventional antibiotics and antimicrobial peptides were performed on both, MSSA and MRSA strains. The conventional antibiotics tested included: ampicillin (Carl Roth GmbH), ciprofloxacin (Fluka), daptomycin (Sigma-Aldrich), erythromycin (Sigma-Aldrich), fusidic acid (Sigma-Aldrich), linezolid (Sigma-Aldrich), lincomycin (Sigma-Aldrich), mupirocin (Sigma-Aldrich), tetracycline (Sigma-Aldrich) and vancomycin (Sigma-Aldrich).

Antimicrobial peptides (AMPs) used in the study were: CAMEL (KWKLFKKIGAVLKVL-NH_2), Citropin 1.1 (GLFDVIKKVASVIGGL-NH_2), LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and Temporin A (FLPLIGRVLSGIL-NH_2). AMPs were synthesized manually by Fmoc chemistry on polystyrene resin modified by Rink Amide linker. Moreover, LL-37 was synthesized on Wang resin due to C-terminal carboxyl group. Deprotection of the Fmoc groups was carried out in 20 min using 20% piperidine in *N*, *N*-dimethylformamide (DMF). Then, the resin was washed with DMF and DCM (dichloromethane) and a chloranil test was performed. All amino acids were coupled using a mixture of DMF/DCM (1 : 1, v/v) in the

presence of coupling agents such as 1-hydroxybenzotriazole (HOBt) and *N*, *N*-diisopropylcarbodiimide (DIC). The degree of acylation was monitored by the chloranil test. The peptides were cleaved from the resin with a mixture consisting of trifluoroacetic acid (TFA), water, triisopropylsilane (TIS) and phenol (92.5 : 2.5 : 2.5 : 2.5, v/v/v/v) as scavengers. In the next step, the peptide compounds were precipitated with cold diethyl ether and lyophilized. All crude products were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) in a gradient of acetonitrile and water, both containing 0.1% TFA. Identity of the peptides was confirmed by mass spectrometry (ESI-MS).

Antimicrobial activity

Minimum inhibitory concentration (MIC) was determined by the broth microdilution method in Mueller Hinton broth according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [14]. Assays for daptomycin were performed in medium supplemented with Ca^{2+} (50 mg/l). Polypropylene 96-well plates with bacteria at initial inoculums of 0.5×10^5 CFU/ml exposed to tested compounds were incubated for 18 h at 37°C. Minimum inhibitory concentration was taken as the lowest concentration of the compound at which visible growth of bacteria was not observed. The experiments were performed in triplicate. Research was conducted in the laboratory of the Department of Inorganic Chemistry, Medical University of Gdańsk.

Table 1. Primers used in PCR amplification of staphylococcal toxin genes

Primer	Sequence of primers (5′–3′)	Size of product [bp]
SEA1	GGT TAT CAA TGT GCG GGT GG	102
SEA2	CGG CAC TTT TTT CTC TTC GG	
SEB1	GTA TGG TGG TGT AAC TGA GC	164
SEB2	CCA AAT AGT GAC GAG TTA GG	
SEC1	AGA TGA AGT AGT TGA TGT GTA TGG	451
SEC2	CAC ACT TTT AGA ATC AAC CG	
SED1	CCA ATA ATA GGA GAA AAT AAA AG	287
SED2	ATT GGT ATT TTT TTT CGT TC	
TSST1-1	ACC CCT GTT CCC TTA TCA TC	326
TSST1-2	TTT TCA GTA TTT GTA ACG CC	
ETA-1	GCA GGT GTT GAT TTA GCA TT	93
ETA-2	AGA TGT CCC TAT TTT TGC TG	
ETB-1	ACA AGC AAA AGA ATA CAG CG	226
ETB-2	GTT TTT GGC TGC TTC TCT TG	
PVL-1	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCCA	433
PVL-2	GCA TCA ACT GTA TTG GAT AGC AAA AGC	

Consideration of whether bacterial isolates are resistant or susceptible was conducted using interpretative criteria provided by CLSI [14].

DNA isolation

High molecular weight, bacterial genomic DNA was purified from lysates with Easy Genomic DNA Preparation (A&A Biotechnology, Poland) as described by Barski *et al.* [15]. Bacterial culture (1 ml) was centrifuged and suspended in 200 µl of 2% Triton X-100 in 2 M NaCl solution (Serva, Germany) and 2–5 µl of Lysostaphin (Sigma-Aldrich, USA) was added to make a final concentration of 25 µg/ml. The mixture was then incubated at 37°C for 30 min, 1 µl of proteinase K (Sigma, USA) was added and the mixture was incubated again at 65°C for 15 min. Subsequently, 400 µl of a chloroform gel (A&A Biotechnology, Poland) was added and the mixture was vortexed for 10 s (vortex, DHN, Poland). The white suspension obtained was centrifuged and the top phase shifted to a new tube. Finally, DNA was precipitated with isopropanol (Serva, Germany). DNA was dissolved in 100 µl of TE buffer (10 mM Tris pH 8.0; 1 mM EDTA; Sigma-Aldrich, USA), and its concentration was determined spectrophotometrically (DNA Calculator, Pharmacia). The DNA was stored at 20°C. Samples were diluted with sterile deionized water to a concentration of 5 ng/µl prior to amplification.

Toxin gene detection by PCR technique

DNA amplification was carried out in a Perkin Elmer 2400 thermocycler (Norwalk, USA). Primers for enterotoxins (SEA, SEB, SEC and SED), TSST-1 and exfoliative toxins (ETA, ETB) were used as described previously [16, 17]. Primers used in PCR amplification of staphylococcal toxin genes are shown in Table 1. Bacterial DNA (50–100 ng) of *S. aureus* isolates was amplified in two sets of multiplex PCR. Set A contained 20 pmol (each) of *sea*, *seb* and *sec* primers and 40 pmol of *sed* primer, while set B contained 50 pmol of *eta* and 20 pmol (each) of *etb* and *tst* primers. Detection of the genes was performed in 50 µl/l of a mixture consisting of: 5 µl/l bacterial DNA (5 ng/µl), 5 µl/l 10× Reaction Buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 0.8% detergent) (MBI Fermentas Lithuania), primers *sea*, *seb*, *sec*, *sed* or *eta*, *etb* and *tsst* (Sigma-Proligo, USA), 200 µM (each) dNTP, 2 U of Taq DNA polymerase (MBI Fermentas Lithuania), 1.5 mM $MgCl_2$. The volume of this mix was adjusted to 50 ml with sterile water. Multiplex primer set B included the same constituents as in set A except for the $MgCl_2$ concentration (2.0 mM) and the primers, which were used at 50 pmol for *eta* and 20 pmol each for *etb* and *tsst*. DNA amplification was carried out in the automatic thermocycler GeneAmp PCR System 2400 (Perkin Elmer, USA) with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, anneal-

ing at 57°C for 2 min, and extension at 72°C for 1 min) and a final extension at 72°C for 7 min. The amplification of PVL genes (*lukS/lukF-PV*) was performed as described by Lina *et al.* [17]. The PCR products were analyzed on 4% agarose gel (Sigma, USA) in the presence of ethidium bromide and photographed under UV illumination. Detection of the PVL genes (*lukS-PV/lukF-PV*) was performed in 50 µl/l of mixture and 1 µl of primers PVL-1 and PVL-2 (100 mM). DNA amplification was carried out with the following thermal cycling profile: 30 cycles of amplification (30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C). The size of amplified fragments was compared with the position of a molecular weight marker (pUC19-DNA/MspI Marker 23; MBI Fermentas, Lithuania) and the reference strains. The negative control consisted of a mixture without the addition of bacterial DNA.

Results

Patients and bacterial isolates

A total of 200 specimens were collected from 100 patients during the study. AD patients consisted of 55% males and 45% females, age 1 to 63 years, median: 22.3 ±15.6 years. *Staphylococcus aureus* was reported in 75 of 100 (75%) skin swabs and 73 of 100 nasal swabs (73%). Six (6%) of the 100 nasal swabs and 5 (5%) of the 100 skin swabs were positive for MRSA. A total of 100 specimens were collected from 50 healthy subjects in the control group. Seven of 50 (14%) nasal swabs and 2 of 50 skin swabs (4%) were positive for *S. aureus*. The MRSA colonization was not reported in the control group. Clinical characteristics are shown in Table 2.

Antibiotic sensitivity pattern of *S. aureus*

The conventional antibiotics used in the study exhibited diverse activities against clinical isolates of *S. aureus*. The vancomycin MIC values were the lowest among the tested antibiotics and varied between 0.125 and 8 µg/ml. The minimal inhibitory concentrations for 90% of isolates (MIC 90) were the lowest for ciprofloxacin and vancomycin. The highest MIC 90 were noted for erythromycin, lincomycin, mupirocin and tetracycline among patients with AD. Similar results were obtained for the control group; see Table 3. Among the conventional antibiotics tested, the highest rates of resistance were observed for ampicillin (58.5%), daptomycin (54.7%), lincomycin (37.5%) and erythromycin (31.0%). The following percentages of resistant strains were noted for: mupirocin 17.5%, tetracycline 15.5%, ciprofloxacin 13.0%. In the control group strains resistant to ampicillin (4%), daptomycin (4%), erythromycin (2%), fusidic acid (1%), lincomycin (3%), and tetracycline (1%) were noted. All strains in the control group were sensitive to ciprofloxacin, linezolid, mupirocin and vancomycin. The antimicrobial resistance patterns (%) of *S. aureus* in patients with atopic dermatitis and the control group are shown in Figure 1. For the antibiotics ciprofloxacin, erythromycin, lincomycin, and tetracycline, the percentage of resistant strains was significantly higher in the MRSA group than the MSSA group (Figure 2). Antimicrobial peptides (CAMEL, Citropin 1.1, LL-37 Temporin A) did not show significant diversity in activity, depending on the tested strain. The concentrations that inhibited growth of *S. aureus* strains were several times higher as compared to those of vancomycin, ciprofloxacin, fusidic acid and linezolid.

Amplification of eta, etb sea, seb, sec, sed, tst and pvl genes

Among tested strains, 17 were positive for *eta*, 5 for *etb*, 3 for *seb* and 21 for *sec*. Seven strains were found to be *tsst* positive, and 16 contained the *pvl gene*. None were positive for *sea* or *sed*. Among MSSA strains greater differentiation of secreted toxins was observed (in order of frequency: sec, *eta*, *pvl*, *tsst*, *etb*, *seb*), while in the group of MRSA strains secretion of 3 toxins (*pvl*, *eta*, *seb*) was noted. Secretion of at least one toxin was observed among MSSA strains in 4 and MRSA strains in 4 cases. Prevalence (%) of staphylococcal toxin genes among tested strains is shown in Table 4. Considering the susceptibility profile, a difference between superantigen positive and negative strains in the group of conventional antibiotics was not noted, except for erythromycin, fusidic acid and mupirocin. Also, we did not find that strains producing tested superantigens were less susceptible to AMPs than nonproducing ones (Table 5).

Discussion

Epidemiology of S. aureus and MRSA strains in AD

Studies worldwide suggest that the prevalence of MRSA in the population with AD varies from 0 to 30.8% [18–20]. In the USA, where CA-MRSA is now the most

Table 2. Clinical characteristics of patients with atopic dermatitis and control group

Parameter	Patients	Control	*P*-value
Total no.	100	50	0.0001[b]
Children	38	2	
Adults	62	48	
Age [years]	22.3 ±15.6	35.1 ±13.4	0.0001[a]
Sex (%):			
Female	45	50	
Male	55	50	
Presence of SA (%):			0.0001[b]
On the skin	75	4	
Anterior nares	73	14	
Presence of MRSA (%):	11	0	
CA-MRSA	54.5%	0	
HA-MRSA	45.5%	0	

[a]*Mann-Whitney U test*, [b]χ^2 *test.*

Table 3. Minimal inhibitory concentration values of *Staphylococcus aureus* strains isolated from atopic dermatitis (AD) patients and control group

Agents	MIC [µg/ml]						*P*-value
	Patients with AD			Control group			
	Range	MIC 50	MIC 90	Range	MIC 50	MIC 90	
Ampicillin	0.125–32	2	16	0.25–32	16	32	0.125584
Ciprofloxacin	0.125–>512	0.5	2	0.125–1	0.25	1	0.2214
Daptomycin	0.125–4	2	4	1–2	2	2	0.6965
Erythromycin	0.125–>512	0.5	> 512	0.125–>512	1	> 512	0.7188
Fusidic acid	0.312–32	0.125	8	0.125–16	1	16	0.0242
Linezolid	0.25–32	1	4	1–4	2	4	0.125059
Lincomycin	0.25–>512	1	64	0.25–>512	0.5	> 512	0.25239
Mupirocin	0.125–>512	0.5	> 512	0.125–32	0.25	32	0.25257
Tetracycline	0.125–64	0.5	32	0.125–32	0.5	32	0.8630
Vancomycin	0.125–8	0.5	2	0.25–1	1	1	0.8069

MIC 50 – MIC for 50% of isolates. MIC 90 – MIC for 90% of isolates. Significant differences between groups determined with Mann-Whitney U test.

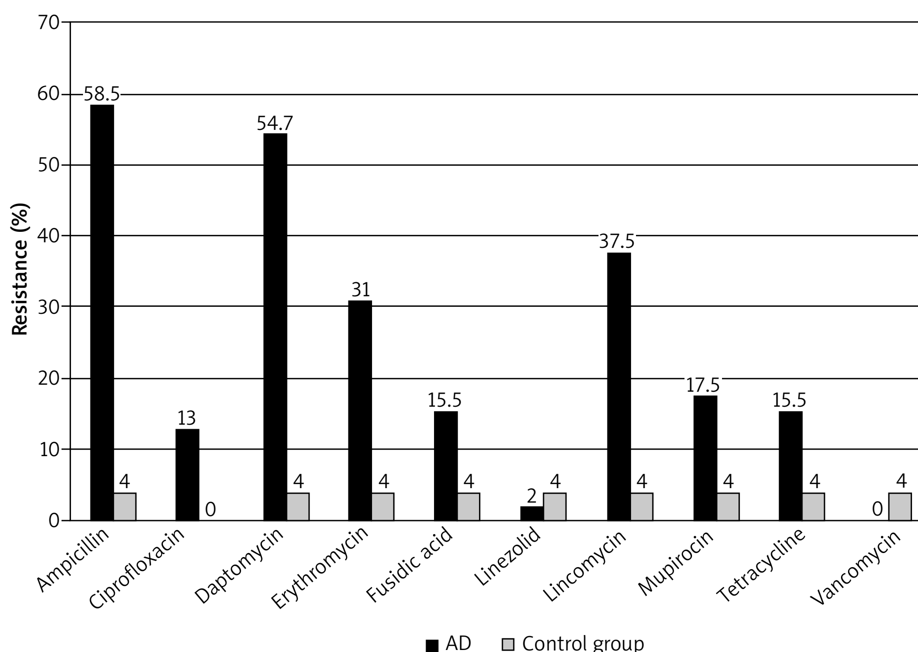

Figure 1. Antimicrobial resistance patterns (%) of *S. aureus* in patients with atopic dermatitis and control group

common pathogen cultured from patients with skin and soft-tissue infections in emergency departments, the colonization rate of AD patients is as high as 18.3% [21]. CA-MRSA strains are responsible for severe infections, including toxic shock, necrotizing fasciitis, and necrotizing pneumonia. These strains have a unique *mecA* cassette (type IV) [22] and have greater antibiotic susceptibility than the HA-MRSA strains [23]. *Staphylococcus aureus* strains were isolated from the majority of our patients, either from the skin (75%) or the anterior nares (73%). In the present study, 6 (6%) of 100 nasal swabs and 5 (5%) of 100 skin swabs were positive for MRSA (55% CA-MRSA, 45% HA-MRSA). Compared with the HA-MRSA strains, CA-MRSA strains also have a higher prevalence of toxin genes, including the enterotoxins, TSST-1, and PVL [24]. Due to the insufficient number of strains, such a relationship in the present study was not observed.

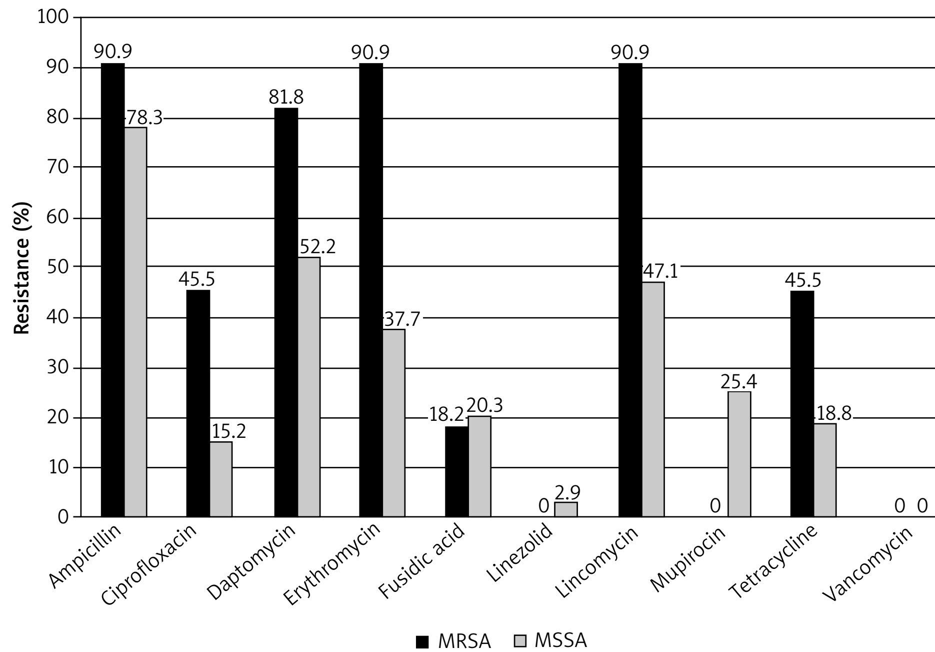

Figure 2. Antimicrobial resistance patterns (%) of *S. aureus* in patients with atopic dermatitis among methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains

Antibiotic resistance

Several studies concerning the effect of antimicrobial treatment on *S. aureus* colonization and the severity of inflammation have given conflicting results. In open or double-blind placebo-controlled trials, topical or systemic antibiotics were able to reduce colonization density and led to a partial improvement of skin lesion [25–27]. In addition, some authors reported that topical steroids in combination with antibiotic treatment can cause the elimination of *S. aureus* from the skin in some patients with AD [28]. On the other hand, treatment with oral antibiotics did not lead to a significant improvement of AD in two double-blind placebo-controlled studies [29, 30]. There are reports suggesting that treatment with antibiotics can temporarily reduce bacterial colonization and improve AD severity. However, these benefits last no longer than 3 months [31]. Antibacterials effective against *S. aureus* include azithromycin, cefuroxime axetil, clarithromycin and erythromycin [32], while CA-MRSA eradication by clindamycin, fusidic acid, trimethoprim-sulfamethoxazole and intranasal mupirocin can be effective. In our study a high rate of resistance to conventional antibiotics was noted. Thirty-one percent of isolated strains were resistant to erythromycin and 15.5% to tetracycline. Our results are comparable with those of Kędzierska *et al*. and Hoeger [33, 34]. In the above-mentioned studies the rate of resistance to erythromycin was respectively 14% and 18%. The increasing resistance to macrolides may suggest that erythromycin no longer should be applied in this indication. In the present study strains resistant to fusidic acid (FA) were also noted. The resistance rate of FA was as high as 15.5%. High rates of fusidic acid (FA)-resistant *Staphylococcus aureus* (FRSA) in patients with skin disease have been previously attributed to high usage of topical FA. The resistance rate of fusidic acid is nearly 10% in the general population and 50% in dermatological patients [35]. Shah *et al.* reported that 50% of *S. aureus* isolates among dermatological patients were resistant to fusidic acid [32]. These data reveal that a high rate of resistance may reveal that fusidic acid should be used in treatment of skin infections only for short periods of time in order to prevent further resistance development. There are reports suggesting that

Table 4. Prevalence (%) of staphylococcal toxin genes among tested strains

Gene	MSSA (*n* = 148)	MRSA (*n* = 11)
eta	12.8% (19)	9.09%
etb	4.7% (7)	0%
pvl	10.1% (15)	27.27%
sea	0% (0)	0%
seb	2.7% (4)	9.09%
sec	15.5% (23)	0%
sed	0% (0)	0%
tsst	6.1% (9)	0%

Table 5. Minimal inhibitory concentration of *Staphylococcus aureus* strains isolated from atopic dermatitis (AD) patients

Agents	MIC [µg/ml]					
	Superantigen positive *Staphylococcus aureus* (*n* = 52)			Superantigen negative *Staphylococcus aureus* (*n* = 48)		
	MIC 50	MIC 90	Range	MIC 50	MIC 90	Range
Ampicillin	1	8	0.125–32	8	16	0.125–32
Ciprofloxacin	0.5	2	0.625–>512	0.25	2	0.0625–>512
Daptomycin	2	2	0.125–4	2	4	0.0625–4
Erythromycin	0.5	> 512	0.125–>512	0.5	256	0.0625–>512
Fusidic acid	0.125	8	0.0625–32	0.125	4	0.0312–32
Linezolid	1	2	0.5–4	1	4	0.25–4
Lincomycin	1	16	0.25–>512	1	64	0.25–>512
Mupirocin	0.5	> 512	0.0625–>512	0.25	512	0.0625–>512
Tetracycline	0.25	32	0.0625–32	0.5	32	0.0625–32
Vancomycin	0.5	1	0.125–2	1	2	0.125–2
CAMEL	4	8	1–8	4	4	2–128
Citropin 1.1	16	16	1–64	16	32	1–128
LL-37	64	256	2–>512	128	> 512	2–>512
Temporin A	8	16	2–64	8	32	4–32

MIC 50 – MIC values for 50% of isolates. MIC 90 – MIC values for 90% of isolates.

short-term use of topical FA for atopic dermatitis has no influence on increasing FRSA [36]. In the present study 17.5% of isolated strains were resistant to mupirocin, which is comparable with other report [37]. Although studies have reported resistance to the newer antimicrobial agents such as linezolid and vancomycin [38], in the present study none of the isolates were resistant to these antibiotics.

Antimicrobial peptides

AMPs seemed to be promising candidates to meet the demand for new classes of anti-infectives with novel mechanism of action. Daptomycin, a lipopeptide antibiotic widely used in the treatment of systemic and life-threatening infections caused by Gram-positive organisms including *S. aureus*, was approved in 2003. Unfortunately, therapeutic failures, relatively uncommon, have been reported [39]. Thus far, the mechanisms underlying daptomycin resistance (DAPR) in *S. aureus* have focused on point mutations in genes involved in phospholipid biosynthesis, particularly *mprF*, which codes for lysyl-phosphatidylglycerol (L-PG) synthetase, *cls2*, which codes for cardiolipin synthase, and *pgsA*, which codes for CDP-diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase [40]. We found 54.8% isolates resistant to daptomycin. The susceptibility breakpoint for daptomycin was considered as < 1 µg/ml for staphylococci as recommended by the CLSI. Reduced susceptibility to vancomycin has been reported to be associated with reduced susceptibility to daptomycin. Diederen *et al.* reported 7 of the 17 vancomycin intermediate *S. aureus* (VISA) isolates to have a daptomycin minimum inhibitory concentration (MIC) of 2 µg/ml and one isolate to have MIC 4 µg/ml [41]. However, such an association was not seen in our study. The observation mentioned above suggests that frequent practice of using daptomycin when vancomycin therapy appears to be failing may be the wrong strategy. Recent data have shown “cross-resistance” between DAP and cationic host defense peptides (HDPs) from neutrophils and platelets in *S. aureus* isolates obtained from patients failing DAP therapy. Similar to many endogenous HDPs, daptomycin contains a significant peptide moiety that can be positively charged by calcium decoration during *in vivo* use. Therefore, one potential driver of such HDP-daptomycin cross-resistance phenotypes may be the capacity of innate HDPs to impact organisms before daptomycin therapy, facilitating increased daptomycin MICs on subsequent daptomycin exposure [42, 43]. Since many of the strains in the present study were isolated from skin infection, it is quite possible to exhibit the daptomycin non-susceptibility during therapy because daptomycin exhibits cross resistance to other cationic host defense peptides. The other antimicrobial peptides (CAMEL, Citropin 1.1, Temporin A) did not show significant diversity in activity LL-37, depending on the tested strain. The concentrations that inhibited growth of SA strains were higher than those of vancomycin, ciprofloxacin, fusidic acid and linezolid.

Virulence factors

Staphylococcus aureus produces numerous virulence factors, including exotoxins such as exfoliatins, Panton-Valentine leukocidin (PVL), superantigens including enterotoxins (SEA-SED), and toxic shock syndrome toxin-1 (TSST-1), that correspond well with our findings (52% of strains excreted tested superantigens). Of the more than 20 staphylococcal enterotoxins, SEA and SEB are the best characterized and are also regarded as superantigens because of their ability to bind to class II MHC molecules on antigen-presenting cells and stimulate large populations of T cells that share variable regions on the β chain of the T cell receptor. The result of this massive T cell activation is a cytokine bolus leading to acute toxic shock [44, 45]. The research regarding the appearance of the enterotoxin genes among the strains of *S. aureus* prove that about half of them have the enterotoxin genes [46]; however, the enterotoxin gene A (*sea*) dominates and about 25% of them have the *tsst* gene [16]. In the present study 2.7% were positive for *seb* and 15.5% for *sec* and none were positive for *sea* or *sed*. Panton-Valentine leukocidin is a cytotoxin that causes leukocyte destruction and tissue necrosis by damage of host defense cells membranes and erythrocytes by the synergistic action of 2 non-associated classes of secretory proteins. The PVL gene has been detected in *S. aureus* strains associated with community-acquired, severe, necrotizing pneumonia and furunculosis [17]. It is produced by fewer than 5% of *S. aureus* strains [46]. Strains isolated from primary necrotic infections involving the dermis, such as furuncles, harbored the PVL genes in 86–93% of cases [17, 47]. In contrast, superficial, non-necrotic processes such as impetigo and folliculitis were not associated with PVL-harboring *S. aureus* [48]. In the present study 10.1% of MSSA and 27.27% MRSA strains secreted PVL. Among MSSA strains greater differentiation of secreted toxins was observed (sec, *eta*, *pvl*, *tsst*, *etb*, *seb*), while in the group of MRSA strains secretion of 3 toxins (*pvl*, *eta*, *seb*) was noted. The purpose of the study was to investigate whether the superantigen positive *S. aureus* strains are more resistant to conventional antibiotics. The effect on toxin production is an important consideration in the selection of an antimicrobial agent to treat staphylococcal infections. Considering the susceptibility profile, a difference between superantigen positive and negative strains in the group of conventional antibiotics was noted for erythromycin, fusidic acid and mupirocin. An alarming observation is the higher resistance of those strains to macrolides which could not only kill bacteria and diminish the rate of colonization but also can suppress the production of superantigen [48]. We did not find that strains producing the tested superantigens were less susceptible to AMPs than nonproducing ones.

Conclusions

Strains of *Staphylococcus aureus* present a significant clinical chalange because of their rising prevalence of antimicrobial resistance. Methicillin-resistant *S. aureus* (MRSA) infections have become a general occurrence in hospitals, and the situation is worrying, since the pathogen is resistant to many antibiotics, including daptomycin and vancomycin, which was considered as the last resort for treatment of MRSA infections. AMPs seemed to be promising candidates to meet the demand for new classes of anti-infectives with a distinctive mechanism of action. Unfortunately, therapeutic failures have been reported, which correspond well with our findings (54.7% of strains resistant to daptomycin). The effect on toxin production is an important consideration in the selection of an antimicrobial agent to treat staphylococcal infections.

Conflict of interest

The authors declare no conflict of interest.

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