Original Article

Potential spread of multidrug-resistant coagulase-negative staphylococci through healthcare waste

Thiago César Nascimento, Vânia Lúcia da Silva, Alessandra B Ferreira-Machado, Cláudio Galuppo Diniz

Department of Parasitology, Microbiology and Immunology, Institute of Biological Sciences, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil

Abstract

Introduction: Healthcare waste (HCW) might potentially harbor infective viable microorganisms in sanitary landfills. We investigated the antimicrobial susceptibility patterns and the occurrence of the *mecA* gene in coagulase-negative *Staphylococcus* strains (CoNS) recovered from the leachate of the HCW in an untreated sanitary landfill.

Methodology: Bacterial identification was performed by physiological and molecular approaches, and minimum inhibitory concentrations (MICs) of antimicrobial drugs were determined by the agar dilution method according to CLSI guidelines. All oxacillin-resistant bacteria were screened for the *mecA* gene.

Results: Out of 73 CoNS, seven different species were identified by 16S rDNA sequencing: *Staphylococcus felis* (64.4%; n = 47), *Staphylococcus sciuri* (26.0%; n = 19), *Staphylococcus epidermidis* (2.7%; n = 2), *Staphylococcus warneri* (2.7%; n = 2), *Staphylococcus lentus* (1.4%; n = 1), *Staphylococcus saprophyticus* (1.4%; n = 1), and *Staphylococcus haemolyticus* (1.4%; n = 1). Penicillin was the least effective antimicrobial (60.3% of resistance; n = 44) followed by erythromycin (39.8%; n = 29), azithromycin (28.8%; n = 21), and oxacillin (16.5%; n = 12). The most effective drug was vancomycin, for which no resistance was observed, followed by gentamicin and levofloxacin, for which only intermediate resistance was observed (22%, n = 16 and 1.4%, n = 1, respectively). Among the oxacillin-resistant strains, the *mecA* gene was detected in two isolates.

Conclusions: Considering the high antimicrobial resistance observed, our results raise concerns about the survival of putative bacterial pathogens carrying important resistance markers in HCW and their environmental spread through untreated residues discharged in sanitary landfills.

Key words: coagulase-negative *Staphylococcus*; antimicrobial resistance; healthcare waste; sanitary landfill; multidrug-resistant bacteria.

J Infect Dev Ctries 2015; 9(1):029-034. doi:10.3855/jidc.4563

(Received 16 December 2013 – Accepted 02 September 2014)

Introduction

Healthcare waste (HCW) is a very important category among the total residues produced nowadays [1]. Besides the physical and chemical characteristics of HCW, its infective potential is a matter of great concern. Historically, literature identifies problems resulting from incorrect HCW management, such as environmental contamination, and accidents involving healthcare professionals and garbage collection personnel. The literature also discusses the spread of infectious diseases among the general population by direct or indirect contact through vectors and water [1]. One of the greatest HCW problems to be addressed is the presence of putative pathogens. The selective pressure of antibiotics and other medicines, as well as chemical compounds commonly discharged as healthcare residues, can lead to the proliferation of these pathogens [2]. These organisms, mainly bacteria, may show antimicrobial resistance and are potential contaminants for hospital surfaces and materials [3]. As they are discharged with untreated residues, these microbial strains may contaminate both the hospital sewer systems and final disposal systems, such as sanitary landfills [3].

As *Staphylococcus* spp., especially oxacillin or methicillin-resistant coagulase-negative strains (CoNS), remain important putative pathogens affecting humans and other animals [4], in this study, we investigated the presence of CoNS in the percolating leachate from the HCW in a Brazilian untreated sanitary landfill. Antimicrobial drug susceptibility patterns of the isolated bacteria were determined and the oxacillin-resistant bacteria were screened for *mecA* since its detection by molecular methods is considered to be of epidemiological importance in characterizing oxacillin resistance among *Staphylococcus* spp. [4]. This study is the first one to isolate and characterize oxacillin-resistant CoNS from HCW in an untreated sanitary landfill.

Methodology

Bacterial samples and 16S rDNA sequencing

One hundred and nine samples of Gram-positive staphylococci were isolated from thirteen 10 mL aliquots of percolating leachate from HCW in a Brazilian sanitary landfill, at Juiz de Fora, Minas Gerais, a southeastern city of 600,000 inhabitants. After serial 10-fold dilutions, the leachate was inoculated in mannitol salt agar (HiMedia Laboratories, Mumbai, India) for selective isolation of *Staphylococcus* spp. The isolated bacteria were presumptively identified as CoNS by morphology after Gram stain and physiological characteristics that included growth in mannitol salt agar, anaerobic glucose fermentation, and a coagulase test. Further species level identification was performed by polymerase chain reaction (PCR) amplification of the specific DNA region codifying for the 16S internal ribosomal RNA from *Staphylococcus* spp. with the primers Staph 756F (5´- AACTCTGTTATTAGGGAAGAACA - 3´) and Staph 750R (5´- CCACCTTCCTCCGGTTTGTCACC - 3´), according to established procedures [5], in an automated thermal cycler (Techne TC-412 Thermal Cycler, Southam Warwickshire, UK). Positive controls were *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus* ATCC 29213, and *Staphylococcus epidermidis* ATCC 12228. Amplified 16S rRNA gene fragments were sequenced by capillary electrophoresis by using an ABI3130 platform (Life Technologies, New York, USA). The electropherograms and sequences were analyzed using Sequence Scanner Software (Applied Biosystems, New York, USA) and BLAST (Basic Local Alignment Search Tool) search function [6].

Antimicrobial susceptibility patterns

The minimum inhibitory concentration (MIC) was determined by the agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. Antibiotic stock solutions were added to melted Mueller-Hinton Agar (HiMedia) to obtain final concentrations ranging from 0.06 to 1,024 µg/mL. The antimicrobial drugs were selected on the basis of microbial characteristics and clinical relevance: penicillin, oxacillin, erythromycin, azithromycin, levofloxacin, gentamicin, and vancomycin (Sigma-Aldrich, Saint Louis, USA). The reference strains *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were included as quality controls.

Screening of the mecA gene

The *mecA* gene was detected by PCR according to the established methodology [5]. The specific primers mecA1 (5´GTAGAAATGACTGAACGTCCGATAA 3´) and mecA2 (5´CCAATTCCACATTGTTTCGGTCTAA 3´) were used, and all the PCR reactions were made in duplicate in an automated thermal cycler (Techne TC-412 Thermal Cycler). Positive and negative controls for the *mecA* gene were included; *Staphylococcus aureus* ATCC 33591 was the positive control, and *Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228 were the negative controls.

Results

Seventy-three bacterial strains isolated from the percolated leachate from HCW were identified at a genus level by PCR amplification of the 16S rDNA. The identification based on 16S rDNA sequencing showed that the species distribution was *Staphylococcus felis* 64.4% (n = 47), *Staphylococcus sciuri* 26.0% (n = 19), *Staphylococcus epidermidis* 2.7% (n = 2), *Staphylococcus warneri* 2.7% (n = 2), *Staphylococcus lentus* 1.4% (n = 1), *Staphylococcus saprophyticus* 1.4% (n = 1), and *Staphylococcus haemolyticus* 1.4% (n = 1).

The results of the antimicrobial drug susceptibility testing are shown in Table 1, and are presented in terms of MIC_{50}, MIC_{90}, and the range of MICs. The antimicrobial susceptibility patterns for the quality control strains *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were in accordance with CLSI guidelines [7]. Penicillin was the least effective drug, with a resistance rate of 60.3% (n = 44), followed by erythromycin (39.8%; n = 29), azithromycin (28.8%; n = 21), and oxacillin (16.5%; n = 12). Vancomycin, levofloxacin, and gentamicin were the most effective antimicrobials. All isolated bacteria were susceptible to vancomycin, and only intermediary resistance was observed against levofloxacin (1.4%, n = 1) and gentamicin (22%, n = 16).

Overall, 23.3% (n = 17) of the tested bacteria were susceptible to all drugs, while 28.8% (n = 21) were resistant to at least one of the tested antimicrobial drugs. Simultaneous resistance to two antimicrobials was observed in 24.6% (n = 18) of the microorganisms, whereas multidrug resistance was observed against three (8.2%; n = 6), four (9.6%; n= 7) and five (5.5%; n = 4) different substances pertaining to the same group of antimicrobial agents and to different groups (Table 2).

Table 1. Antimicrobial susceptibility patterns of the coagulase-negative *Staphylococcus* spp. isolated from the percolated leachate in a sanitary landfill from the city of Juiz de Fora, Minas Gerais, Brazil

Antimicrobials	MICs (µg/mL)			% S (n)	% IR (n)	% R (n)
	50%	90%	Range			
Penicillin	0.25	>1,024	0.06 – >1,024	39.7 (29)	-	60.3 (44)
Oxacillin	0.25	>1,024	0.06 – >1,024	83.5 (61)	-	16.5 (12)
Erythromycin	0.5	64	0.06 – >256	48 (35)	12.2 (9)	39.8 (29)
Azithromycin	1	16	0.06 – 16	67.1 (49)	4.1 (3)	28.8 (21)
Levofloxacin	0.25	1	0.06 – 2	98.6 (72)	1.4 (1)	-
Gentamicin	0.25	8	0.06 – 8	78 (57)	22 (16)	-
Vancomycin	1	2	0.06 – 4	100 (73)	-	-

S: sensitivity; IR: intermediate resistance; R: resistance

Table 2. Resistance phenotypes and *mecA* detection among *Staphylococcus* spp. isolated from untreated HCW

Species (n)	Resistance phenotype	*mecA*	Frequency (%)	n
S. felis (47)	AZI, ERY, GEN, LEV, PEN	-	2.1	1
	AZI, ERY, OXA, PEN	-	2.1	1
	AZI, GEN, OXA, PEN	+	2.1	1
	AZI, ERY, PEN	-	2.1	1
	AZI, GEN, PEN	-	4.2	2
	AZI, OXA, PEN	+	2.1	1
	AZI, PEN	-	2.1	1
	ERY, GEN	-	10.6	5
	ERY, PEN	-	19.1	9
	AZI	-	4.2	2
	ERY	-	6.4	3
	PEN	-	19.1	9
S. sciuri (19)	AZI, ERY, GEN, OXA, PEN	-	5.2	1
	AZI, GEN, OXA, PEN	-	5.2	1
	AZI, ERY, GEN, PEN	-	5.2	1
	AZI, ERY, OXA, PEN	-	10.4	2
	AZI, OXA, PEN	-	5.2	1
	AZI, PEN	-	5.2	1
	OXA, PEN	-	5.2	1
	PEN	-	26.3	5
S. epidermidis (2)	AZI, ERY, GEN, OXA, PEN	-	50	1
	OXA, PEN	-	50	1
S. warneri (2)	ERY	-	50	1
	GEN	-	50	1
S. lentus (1)	AZI, ERY, GEN, OXA, PEN	-	100	1
S. saprophyticus (1)	AZI, ERY, PEN	-	100	1
S. haemolyticus (1)	AZI, ERY, GEN, PEN	-	100	1

AZI: azithromycin; ERY: erythromycin; GEN: gentamicin; LEV: levofloxacin; OXA: oxacillin; PEN (penicillin).

Among the oxacillin-susceptible *Staphylococcus*, the *mecA* gene was not detected. However, of the oxacillin-resistant strains, the *mecA*$^+$ genotype was observed in only two isolated bacteria identified as *S. felis*. Although the *mecA* gene was not present in most of the oxacillin-resistant staphylococci (n = 10), when comparing the oxacillin susceptibility patterns, a high heterogeneity was observed. MICs for oxacillin varied between 0.5 and > 1,024 μg/mL (MIC_{50} = 0.5 μg/mL; MIC_{90} > 1,024 μg/mL) among the *mecA*$^-$ resistant strains. Among the *mecA*$^+$ bacteria, the MICs for oxacillin were recorded as 0.5 and 1.0 μg/mL.

Discussion

Of the CoNS isolated from the percolated leachate from the HCW in the sanitary landfill, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus* are frequently associated with human diseases such as infections associated with intravenous catheters, osteomyelitis, endocarditis, and renal and skin infections [8]. Other species also identified, such as *S. sciuri*, *S. lentus*, and *S. vitulinus*, are widely distributed in environment, mainly in food, farm animals, rodents, marsupials, and water mammals, but recently have been associated with severe human infections such as endocarditis, peritonitis, septic shock, infections of the urinary tract, and open wounds [4]. *Staphylococcus lentus* is a commensal bacterium colonizing the skin of several animal species. It has commonly been isolated from food-producing animals, including poultry and dairy livestock [9]. In dairy sheep and goats, *S. lentus* has been associated with subclinical mastitis [10], and has rarely been associated with human diseases [11,12]. *S. felis* has been associated with skin infections and otitis in cats [13]. In recent years, septic arthritides due to *S. warneri* have been reported, mostly as opportunistic colonization in patients with prosthetic devices [14].

Based on the antimicrobial susceptibility patterns observed, resistance against penicillin, erythromycin, azithromycin, oxacillin, and intermediary resistance against gentamicin, are worrisome. According to the literature, the occurrence of resistant bacteria in open environments is significant not only as an indication that resistant microorganisms are circulating, but also for issues related to the dissemination of genetic markers [15,16]. According to some authors, little is known about the antibiotic resistomes [17,18] (*i.e.*, the collection of all the antibiotic resistance genes and their precursors) in pathogenic and non-pathogenic bacteria.

Recent studies have shown that oxacillin-resistant CoNS isolated from clinical specimens may also be resistant to erythromycin, clindamycin, and ciprofloxacin. Especially when penicillin is contraindicated, erythromycin has been extensively prescribed for antimicrobial therapy [19]. In this study, intermediate resistance was observed against levofloxacin and gentamicin. Cross-resistance between oxacillin, aminoglycosides, and quinolones has already been reported [20]. As expected, no resistance was observed against vancomycin, which is widely accepted as the most effective drug to treat staphylococcal infections [4,20].

Overall, the finding of multiple antimicrobial resistances in CoNS is alarming, particularly if the genes encoding these phenotypes are available for transfer to other pathogens, or if humans and other animals get contaminated with these resistant bacteria. The potential factors that might be associated with the selective pressure resulting in multiple resistances were not explored, but one of them may be co-selection, as suggested by phenotypic evidence found in other studies [16]. The prevalence of resistant bacteria in the environment, such as the untreated sanitary landfill evaluated, inspire hypotheses about the native roles of so-called resistance genes in different microbial communities, enforcing the need for more detailed studies on environmental reservoirs of resistance [17].

In general, Brazilian hospitals are not required to perform species-level identification of the so-called putative bacterial species; within the *Staphylococcus* genera, only *S. aureus* identification is performed and susceptibility patterns are recorded. For coagulase-negative *Staphylococcus* spp., antimicrobial susceptibility is performed only if the bacterium is related to patient infections, but bacteria samples are not kept in hospital laboratories. The results showed in this study would be of high relevance to support alterations in healthcare regulations to avoid the environmental contamination with multidrug-resistant bacteria from HCW.

The *mecA* gene, which codifies the synthesis of penicillin-binding proteins (PBP) PBP2a or PBP2′ having low affinity to other β-lactam antimicrobials besides oxacillin, was detected in only two of the oxacillin-resistant strains. The gene is inserted in a mobile genetic element, SCC*mec*, which is of fundamental importance in the transmission and epidemiology of bacterial resistance [21].

Detection of *mecA* by molecular methods is considered the gold standard in the characterization of oxacillin resistance. It should be noted that all *mecA*$^+$ strains are reported to be oxacillin resistant. However,

oxacillin interpretative criteria may overcall resistance for *mecA*$^-$ strains with MICs for oxacillin between 0.5 and 2.0 µg/mL. Exclusively found in oxacillin-resistant staphylococci, no allelic equivalent to the *mecA* gene was described in oxacillin-susceptible strains, although other mechanisms may interfere with oxacillin resistance in both *mecA*$^+$ and *mecA*$^-$ staphylococci [22]. In this regard, resistance to oxacillin may be extrinsic, non-*mecA* mediated, known as borderline [23-25]. According to the literature, the borderline phenotype may be related to excessive production of β-lactamases [23,24]. Additionally, that borderline phenotype may also be attributed to other mechanisms, such as production of plasmid-mediated inducible oxacillinase, or spontaneous amino acid substitution in the transpeptidase domain due to mutations in PBP genes [25].

Conclusions

As a matter of concern, our results raise issues related to the viability of putative pathogenic bacteria resistant to important antimicrobial drugs carrying important resistance markers in untreated HCW in sanitary landfills. Communities and the entire environment surrounding these disposal areas may be at risk if these and other viable microorganisms cross the contention barriers. These risks regarding the potential spread of leachate from sanitary landfills due to human and animal activities, or even due to weather phenomena, such as torrential rains and floods, should be considered. Our results address a phenomenon related to the incorrect HCW management in Brazil and in other geographical regions. Taking into account environmental health, more conscientious policies should be considered by authorities to avoid the disposal of HCW waste without any further treatment.

Acknowledgements

The authors are grateful to the Departamento Municipal de Limpeza Urbana de Juiz de Fora (DEMLURB) and Programa de Pós-graduação em Saúde (PPGS/UFJF).

This work was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Authors' contributions

T. C. Nascimento and A. B. Ferreira-Machado contributed to the experimental data collection and analyses; V. L. Silva and C.G. Diniz contributed to the experimental design, funding and data analyses.

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Corresponding author

Professor Cláudio Galuppo Diniz, PhD
Laboratory of Bacterial Physiology and Molecular Genetics,
Department of Parasitology Microbiology and Immunology,
Institute of Biological Sciences, Federal University of Juiz de Fora, 36.036-900, Juiz de Fora, MG, Brazil
Phone/Fax: + 55 32 2102-3213
Email: claudio.diniz@ufjf.edu.br

Conflict of interests: No conflict of interests is declared.