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ORIGINAL ARTICLE

Phenotypic and genotypic characterization of clinically relevant bacteria isolated from dental waste and waste workers' hands, mucosas and coats

T.L. Tagliaferri[1], C.D. Vieira[1], M.A.R. de Carvalho[1], L.C.D. Ladeira[2], P.P. Magalhães[1], L. de Macêdo Farias[1] and S.G. dos Santos[1]

1 Department of Microbiology, Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil
2 Nuclear Technology Development Center (CDTN), Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil

Significance and Impact of the Study: Infectious dental waste can contain clinically relevant bacteria with important resistance and biofilm profiles. These micro-organisms could be transmitted to waste workers, other professionals and patients if the principles of biosafety measures are neglected. To our knowledge, no study has ever evaluated the microbial characterization and the potential contamination risk of dental infectious waste and waste handlers. The presence of clinically relevant bacteria in the hands and nasal mucosa of waste workers highlights the need for studies in this field to clarify the risk of these pathogens in dental healthcare services, and to stress the need for an efficient waste management.

Keywords
infectious waste, clinically relevant bacteria, infectious dental waste, biosafety, waste workers, contamination, resistance, biofilm.

Correspondence
Simone Gonçalves dos Santos, Laboratório de Microbiologia Oral e de Anaeróbios, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. Avenida Presidente Antônio Carlos, 6627, C.P. 486, Pampulha - Campus UFMG, 31270-901 Belo Horizonte, MG, Brasil.
E-mail: simonesantoskey@ufmg.br

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Abstract

Infectious wastes are potential sources of pathogenic micro-organisms, which may represent a risk to the professionals who manage them. In this study, we aimed to characterize the infectious bacteria present in dental waste and waste workers. The dental waste produced over 24 h was collected and waste workers were sampled by swabbing. Isolate resistance profiles were characterized by Vitek® and PCR and biofilm formation by Congo Red agar, string test and microtitre assay. To assess similarity between the waste and the workers' samples, a random amplified polymorphic DNA test was used. Twenty-eight bacteria were identified as clinically relevant. The most frequent gene was bla_{TEM} present in five Gram-negative micro-organisms, and one bla_{SHV} in *Klebsiella pneumoniae*. All *Pseudomonas aeruginosa* were positive to extracellular polymeric substances formation, except one isolated from a worker. *Klebsiella pneumoniae* had negative results for the string test. *Pseudomonas aeruginosa* showed better adherence at 25°C after 48 h of incubation and *K. pneumonia* had the best biofilm formation at the same temperature, after 24 h. The similarity between *P. aeruginosa* recovered from dental waste and from workers was low, however, it is important to note that a pathogen was found on a worker's hands and that improvements in biosafety are required.

Introduction

Waste handlers are more susceptible to infections caused by pathogenic micro-organisms present in sharp materials, in bioaerosol or by the contact of micro-organisms with skin and mucosa, than other healthcare professionals (Hossain *et al.* 2013; WHO 2014). According to the World Health Organization (2014), the transmission of these bacteria occurs through a 'chain of infection' comprising six stages: (i) the presence of an infectious agent, (ii) a reservoir, (iii) a portal of exit, (iv) the mode of transmission, (v) a portal of entrance and (vi) a susceptible host. Although the biosafety control measures for waste handlers are mostly directed at the mode of transmission, breaking any link in the chain can prevent infection acquisition.

Clinically relevant micro-organisms such as those from the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.), *Escherichia coli* and other important species were already isolated in dental solid waste and may represent a risk for waste handlers, since these bacteria are commonly involved in nosocomial infections and have a high antimicrobial resistance rate (Rice 2008; Vieira *et al.* 2011). Once they are present on workers' hands, they can be disseminated, and may colonize biotic and abiotic surfaces through biofilm formation. Once the cross-contamination chain has begun through incorrect waste management or lack of biosafety procedures, other healthcare workers, as well as patients, could be affected.

In this study, we aimed to isolate and characterize the infectious bacterial agents present in dental waste and waste handlers and to evaluate whether professionals are serving as transmission vehicles. Knowledge of biofilm production and resistance profiles can help to improve institutional waste management practices and the biosafety procedures of workers, not only for their own protection but also to avoid environmental dissemination of these micro-organisms and the cross-contamination of patients and healthcare professionals (Rice 2008; Hossain *et al.* 2013). This study is motivated by the lack of data relating to the biosafety of healthcare waste workers and the characterization of clinically relevant micro-organisms in dental solid waste and those professionals who manage it.

Results and discussion

Bacteria isolation in waste and workers

In this study, 345 different morphotypes were collected from workers and 16 from infectious dental waste, of which 320 were Gram-positive cocci, 11 Gram-positive rods and 30 Gram-negative rod bacteria. Twenty-eight of the 361 isolated bacteria were identified as clinically relevant and are specified in Tables 1 and 2. The presence of clinically relevant micro-organisms in waste handling workers highlights their role as potential vehicles of pathogen transmission and suggests that biosafety procedures could be neglected.

In developing countries, knowledge of the potential risks of infectious waste is still low among healthcare professionals, mainly because of the lack of studies investigating the role of infectious waste as a potential pathogen reservoir, and its capacity to cause infections. Clinical staff is also unaware of biomedical waste management, as well as the risk of micro-organism contamination (Hossain *et al.* 2011).

Research conducted in India showed that dentists are inadequately informed about the relevant infectious agents in dentistry, including *P. aeruginosa* and other important clinical bacteria. These professionals do not attend waste management training, which results in incorrect segregation and potential cross-contamination by waste handlers (Singh *et al.* 2012).

Pseudomonas aeruginosa was the most frequently isolated Gram-negative bacteria in this study, and the only species found both on waste workers' hands and in dental infectious waste. Vieira *et al.* (2011) also detected the presence of this genus and other clinically relevant bacteria isolated in this study such as *Klebsiella* spp., *Acinetobacter* spp., *Escherichia* spp. and *Staphylococcus* spp. in infectious waste at the same institution in 2011. The presence of some of these pathogens on waste workers, especially on their hands, could be hazardous, considering that these micro-organisms can be part of an infection chain, affecting other individuals such as the cleaning staff, who have a higher incidence of acquired infectious diseases when compared to other professionals, due to the nature of their work (Hossain *et al.* 2013).

Pseudomonas aeruginosa can be recovered from dental instruments and equipments, such as air/water syringes, high-/low-speed drills. The species has been identified in high proportion in the subgengival biofilms of patients with periodontal diseases (Oliveira *et al.* 2008; Colombo *et al.* 2013). This finding is highlighted by the fact that waste professionals are serving as vehicles of bacterial transmission, favouring the contamination of dental environment and also increasing the risk of patients' infection by this opportunistic pathogen.

Antimicrobial resistance

Antimicrobial and gene resistance profiles are shown in Tables 1 and 2. All Gram-negative strains had negative results for the $bla_{\text{CTX-M}}$, bla_{IMP}, bla_{GIM}, bla_{SPM}, bla_{VIM} genes and no *Staphylococcus aureus* had a *mec*A gene.

According to Magiorakos *et al.* (2012) many authors consider multidrug-resistant (MDR) micro-organisms as those resistant to three or more classes or subclasses of antimicrobial agents. As shown in Table 1, all *P. aeruginosa* strains isolated were classified as MDR and showed the same antimicrobial profile, which includes resistance to penicillin, cephalosporins and glycylcyclines. In recent years, rates of resistance to third-generation cephalosporin have been increasing in this species, and the present results are in agreement with the literature (Hirsch and Tam 2010). Two *E. coli* strains isolated in our study had positive results for cephalosporin resistance, and intermediate or resistance classification for carbapenems and colistin. The emergence of these *E. coli* strains raises great

Table 1 Identification and determination of susceptibility profile of Gram-negative micro-organisms recovered from infectious waste and workers

	Gram-negative																	
			Antimicrobial tested														Resistance genes	
Micro-organism	Isolation place	Worker	AMI	GEN	AMP	SBA	CPM	CTX	CAZ	CRO	CXM	CIP	CL	ETP	IMI	MER	TIG	bla_{TEM}	bla_{SHV}
1. *Escherichia coli*	NM	07	S	S	I	S	S	R	R	R	R	S	R	I	S	S	S	POS	NEG
2. *Escherichia coli*	NM	07	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NEG	NEG
3. *Escherichia coli*	NM	07	S	S	I	I	S	S	R	R	R	S	R	R	I	R	S	NEG	NEG
4. *Escherichia coli*	NM	07	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NEG	NEG
5. *Escherichia coli*	NM	07	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	NEG	NEG
6. *K. pneumoniae*	Hands	07	S	S	R	S	S	S	S	S	S	S	S	NE	S	S	S	POS	POS
7. *Pseudomonas aeruginosa*	Hands	07	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	NEG	NEG
8. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	NEG	NEG
9. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	NEG	NEG
10. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	POS	NEG
11. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	NEG	NEG
12. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	POS	NEG
13. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	NEG	NEG
14. *Pseudomonas aeruginosa*	Waste	–	S	S	R	R	S	R	S	R	R	S	S	NE	S	S	R	POS	NEG
15. *Acinetobacter junii*	Waste	–	S	S	S	S	S	S	S	S	S	S	R	NE	S	NE	S	NEG	NEG
16. *Acinetobacter junii*	Waste	–	S	S	S	S	S	S	S	S	S	S	S	NE	S	NE	S	NEG	NEG

NM, Nasal Mucosa; AMI, Amikacin; GEN, Gentamicin; AMP, Ampicillin; SBA, Ampicillin/Sulbactam; CPM, Cefepime; CTX, Cefoxitin; CAZ, Ceftazidime; CRO, Ceftriaxone; CXM, Cefuroxime; CIP, Ciprofloxacin; CL, Colistin; ETP, Ertapenem; IMI, Imipenem; MER, Meropenem; TIG, Tigecycline; R, Resistant; I, Intermediate Resistance; S, Sensitive; NE, Nonevaluated; POS, Positive; NEG, Negative.

Table 2 Identification and determination of susceptibility profile of Gram-positive micro-organisms recovered from infectious waste and workers

	Gram-positive																	
			Antimicrobial tested															Resistance gene
Micro-organism	Isolation place	Worker	BZP	OXA	GEN	CIP	MXF	NOR	ERY	CLI	LNZ	TEC	VAN	TIG	FA	RFP	SXT	*blaZ*
17–28 *Staphylococcus aureus*	NM	07,08,11	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	POS

NM, Nasal Mucosa; BZP, Benzylpenicillin; OXA, Oxacillin; GEN, Gentamicin; CIP, Ciprofloxacin; MXF, Moxifloxacin; NOR, Norfloxacin; ERY, Enythromycin; CLI, Clindamycin; LNZ, Linezolid; TEC, Teicoplanin; VAN, Vancomycin; TIG, Tigecycline; FA, Fusidic Acid; RFP, Rifampicin; SXT, Trimethoprim/Sulfamethoxazole; R, Resistant; S, Sensitive; POS, Positive.

concern, since these antimicrobial treatments are the last resort with which to treat serious infections of Enterobacteriaceae (Yao *et al.* 2016).

The most common gene found among our strains was bla_{TEM}, which was present in five Gram-negative microorganisms (31·25%), while only one bla_{SHV} gene was detected in *K. pneumoniae*. A study conducted in Iran assessed ESBLs in *P. aeruginosa* and had similar results, with the TEM enzyme being the most prevalent in the isolated samples (Bokaeian *et al.* 2015). Bali *et al.* (2010) evaluated the presence of $bla_{TEM,}$ bla_{SHV} and $bla_{CTX\text{-}M}$ in *E. coli* and *K. pneumoniae* isolated from clinical specimens. The authors found a high prevalence of the bla_{TEM} gene and a low number of $bla_{CTX\text{-}M}$ in their samples, and detected the carrying of mutual genes in a single strain, also reported by the present study. The *blaZ* gene is also disseminated in *S. aureus* from Chong's study. The authors collected a total of 220 *S. aureus* from bacteraemia and observed the gene presence in 92% of their strains (Chong *et al.* 2015).

In this study, *K. pneumoniae* had positive results for the bla_{TEM} and bla_{SHV} genes, but revealed a phenotype of resistance only to penicillin. According to Pellegrino *et al.* (2008) and Picão *et al.* (2012), the combination of many mechanisms may be necessary to achieve antimicrobial resistance, or these samples may even exhibit a higher permeable membrane, allowing drug action.

Biofilm formation

All *P. aeruginosa,* except the one from a worker's hands, had positive results for EPS formation. *K. pneumoniae* had a negative result for the string test. In most of the evaluated occurrences, the *P. aeruginosa* isolated from the worker's hands was weakly adherent at 25°C, a temperature in which 87·5% of those isolated from infectious waste were classified as strongly adherent after 48 h. The EPS results are consistent with biofilm formation as this substance is responsible for its physicochemical integrity (Gellatly and Hancock 2013). *K. pneumoniae* isolated in worker's hands were also strongly adherent at 25°C, but in a shorter period of incubation, of only 24 h.

A similar result was found by Pedrosa *et al.* (2014) who showed that *P. aeruginosa* isolated from natural mineral water were more adherent after a 25°C incubation. Zeraik and Nitschke (2012) described a pattern where biofilm adhesion increased as temperature decreased, suggesting that temperature stress stimulates biofilm production. The biofilm production at room temperature by *P. aeruginosa* and *K. pneumoniae* highlights the importance of the daily cleaning of medical devices and healthcare surfaces to avoid the establishment of a strongly adherent biofilm on these surfaces by clinically relevant bacteria. As

shown in the present study, *P. aeruginosa* and *K. pneumonia* have the potential to form biofilms, which constitutes an important threat to biosafety, since micro-organisms can adhere to surfaces, forming a pathogen and resistance gene reservoir and a source of cross-contamination, especially on professionals' hands, affecting themselves, other staff and patients (Abdallah *et al.* 2014).

Similarity patterns

The similarity patterns in the strains revealed three different clusters in *P. aeruginosa*: cluster one presenting six waste strains, and two other clusters represented by one strain isolated from infectious waste (cluster two) and another from the worker's hands (cluster three) as shown in Figure 1.

The clonal similarity among *P. aeruginosa* strains was assessed using RAPD, which characterized 62·5% as being the same clone, although with divergent phenotypic characteristics. The hand strain shows low genetic similarity to the waste strains, so we cannot prove direct cross-contamination. More studies are required to understand the spread of pathogens and infectious diseases in clinical waste workers, as the current literature is still limited on that subject (Hossain *et al.* 2013). Although the origin of the micro-organism is still unknown, its presence on the waste handler's hand was demonstrated. Based on these results, it is possible to infer that biosafety procedures may have been ignored by waste workers in the institution.

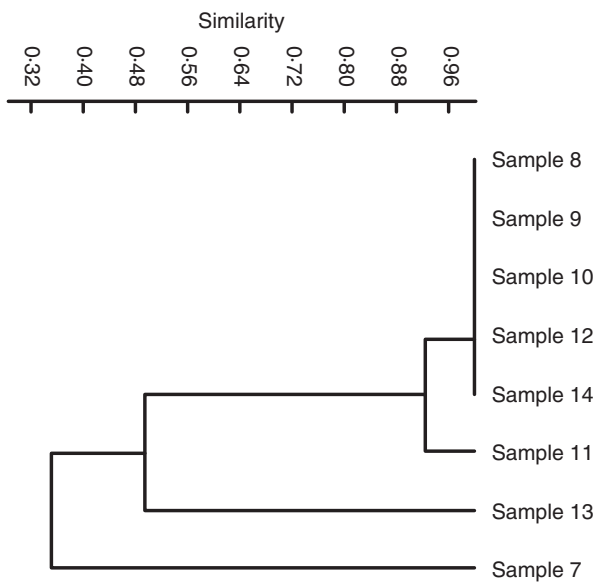

Figure 1 Dendrogram of evaluated *Pseudomonas aeruginosa* strains isolated from infectious waste and worker's hands. Samples were grouped in Cluster number I, containing the strains 8–12 and 14; Cluster II, comprising the strain 13 and Cluster III, consisting of strain number 7. All samples were isolated in infectious waste, except sample 7, as shown in Table 1.

This study confirmed the presence of clinically relevant bacteria in healthcare waste workers, who can serve as pathogen transmission vehicles affecting other professionals and patients. *S. aureus*, *E. coli* and *K. pneumoniae* isolated in workers' hands and nasal mucosa had positive results for resistance genes and *P. aeruginosa* recovered in infectious waste. *K. pneumoniae* and *P. aeruginosa* isolated in professional's hands are able to form adherent biofilm at 25°C, confirming the need for constant cleaning of healthcare surfaces to avoid bacteria attachment and their dissemination. Although this study could not prove the origin of the clinically relevant micro-organisms present in workers, the findings suggest that biosafety procedures are being ignored, which may be the cause of professional contamination by bacteria with a concerning resistance and virulence profile.

Materials and methods

Institutional characteristics

Waste and waste worker sampling took place at a Public Dental Health Care Service in Belo Horizonte, Brazil. The clinical staff consisted of 215 workers offering consultations in several dental specialties. The team responsible for processing environmental surfaces and waste collection consisted of 12 members of staff. All professionals attended semi-annual training programmes on infection control.

This work was approved by the Ethics Committee on Human Research of the Hospital of the Military Police of Minas Gerais (006/2013), and was also approved by the Research Ethics Committee of UFMG, number 24911213.5.0000.5149. Informed consent was signed by all volunteers.

Identification and characterization of bacteria strains

The microbial content of 12 workers was evaluated in two different periods using samples of nasal mucosa and professional coats, according to the methodology of Snyder *et al.* (2008). Hands were sampled by swabbing the dorsum of each finger, finishing with two circles across the palms in a swirling motion. Only one swab was used for both hands, starting with the nondominant one. Dental infectious waste produced over 24 h was collected and quartered based on Tiew *et al.* (2010) and a random aliquot of 500 g was collected and left in sterile saline for 1 h to assess the microbial content of the leached liquid. The samples were spread over enriched and selective

mediums and, after morphological and biochemical screening, the Gram-negative micro-organisms and those Gram-positive with positive results for catalase, coagulase and DNase tests, were identified and had their antimicrobial susceptibility profile investigated using the automated system Vitek® 2 Compact (Biomerieux, Marcy I' Etoile, France).

To verify whether Gram-negative strains were extended spectrum beta lactamase (ESBL) and carbapenemase reservoirs, the presence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{IMP}, bla_{GIM}, bla_{SPM} and bla_{VIM} genes was evaluated, as described previously (Paterson *et al.* 2003; Jones *et al.* 2009; Findlay *et al.* 2012). The resistance genes *mec*A and *blaZ* from Gram-positive bacteria were evaluated according to preceding studies (Martineau *et al.* 2000; Al-Charrakh and Obayes 2014). The DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega®, Madison, WI).

To assess clone similarity among *P. aeruginosa* strains found in worker's hands and in infectious waste, and to verify the presence of cross-contamination, a random amplified polymorphic DNA (RAPD) test was performed as described by Drabrowsky *et al.* (2003). Similarity was determined using Dice coefficients, and a dendrogram was constructed using PAST 3.0. profiles, where more than 85% similarity were considered genetically related.

To evaluate the capacity of the isolated micro-organisms from worker's hands to develop biofilm, EPS production, hypervirulent phenotype and biofilm adherence were assessed. Strains from the same species found in the infectious waste were also assessed to compare adherence among them. The Congo Red Agar (CRA) method was used to evaluate EPS production based on Ferreira *et al.* (2014). The string test was used to assess hypervirulence according to the protocol of Shon *et al.* (2013). The microtitre plate assay was based on the protocol of Perez *et al.* (2011). Biofilm adherence was classified based on the optical density of negative controls (OD_c) into the following groups: $OD \leq OD_c$ = nonadherent; $OD_c < OD \leq 2xOD_c$ = weakly adherent; $2xOD_c < OD \leq 4xOD_c$ = moderately adherent; $4xOD_c < OD$ = strongly adherent.

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Conflict of Interest

No conflict of interest declared.

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