Antibiotic resistance in coagulase negative staphylococci isolated from wastewater and drinking water

Cátia Faria [a], Ivone Vaz-Moreira [a,b], Eduarda Serapicos [a,b], Olga C. Nunes [b], Célia M. Manaia [a,*]

[a] CBQF, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal

[b] LEPAE, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, 4200-465 Porto, Portugal

Keywords:
Coagulase-negative staphylococci
Antibiotic resistance
Drinking water
Erythromycin
*msr*A

A B S T R A C T

This study reports the antibiotic resistance patterns of coagulase negative staphylococci (CNS) isolated from a drinking water treatment plant (WTP), a drinking water distribution network, responsible for supplying water to the consumers (WDN), and a wastewater treatment plant (WWTP), responsible for receiving and treating domestic residual effluents. Genotyping and the 16S rRNA gene sequence analysis demonstrated a higher diversity of species both in the WTP (6 species/19 isolates) and WWTP (12 species/47 isolates) than in the WDN (6 species/172 isolates). *Staphylococcus pasteuri* and *Staphylococcus epidermidis* prevailed in the WTP and WDN and *Staphylococcus saprophyticus* in the WWTP. Staphylococci with reduced susceptibility (resistance or intermediary phenotype) to beta-lactams, tetracycline, clindamycin and erythromycin were observed in all types of water and belonged to the three major species groups. The highest resistance rate was found against erythromycin, presumably due to the presence of the efflux pump encoded by the determinant *msr*A, detected in the majority of the resistant isolates. This study demonstrates that antibiotic resistant CNS may colonize different types of water, namely drinking water fulfilling all the quality standards.

Introduction

Staphylococci are ubiquitous bacteria reported as part of the normal microbiota of air, soil, water, humans and other animals, and processed food products as cheese or sausages (Kloos et al., 1992; Norton and LeChevallier, 2000; Smith et al., 2001; Iacumin et al., 2006; Irlinger, 2008). Their cell structure of Gram-positive cocci is responsible for a high tolerance to dryness, dehydration and low water activity, and explains in part their widespread distribution and persistence in the environment (Kloos et al., 1992).

As many other ubiquitous bacteria, some coagulase-negative staphylococci (CNS) may behave as opportunistic pathogens, often introduced by medical devices or colonizing exposed wounds (Huebner and Goldmann, 1999; Dowd et al., 2008; Irlinger, 2008). Due to the intensification of invasive medical practices and increased numbers of immuno-deficiencies, the risks of infection by CNS had increased during the last years (Rupp and Archer, 1994). Thus, given their modes of transmission, CNS are highly undesirable contaminants of the clinical environment (e.g. air, water, biofilms). Moreover, the hazards associated with CNS can be worsened by the notorious escalade of antibiotic resistance observed during the last decades in these bacteria (John and Harvin, 2007; Irlinger, 2008). Even though CNS opportunistic infections are most of the times regarded as of environmental origin, the distribution and antibiotic resistance patterns of these organisms in the environment are poorly characterized.

Microbial aquatic ecosystems, mainly those integrating the urban water cycle, represent important vehicles for the dissemination of human-associated microorganisms. During this study, the diversity of CNS and the respective antibiotic resistance trends were assessed in different types of water — 1) a drinking water treatment plant, where raw water is collected and treated (WTP); 2) a water distribution network, that receives treated water and supply it to the consumers (WDN); and 3) a wastewater treatment plant (WWTP), where domestic residual effluents are treated and discharged into the environment, re-entering into the natural water courses. The physicochemical and biotic characteristics vary with the different types of water and hence a diverse microbiological pattern is expected at different sites of this circuit (Norton and LeChevallier, 2000; Eichler et al., 2006). The present study aimed at assessing the taxonomic diversity and antibiotic resistance trends in CNS thriving in these different types of water.

Materials and methods

Water samples and analysis

Samples were collected in three different sites, corresponding to distinct stages of the urban water circuit. WTP samples were collected from three different sites of a water treatment system in a single sampling campaign in December 2007. This facility is responsible for

* Corresponding author. Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal. Tel.: +351 22 5580059; fax: +351 22 5090351.
E-mail address: cmmanaia@esb.ucp.pt (C.M. Manaia).

the water-supply of about 1.5 million of habitants, where the raw water from a river basin is treated by ozonization, flocculation and chlorination. The WDN has a total storage capacity of 130 000 m^3, and a distribution network of 730 km. Samples were collected at 33 sampling points defined for the routine monitoring analysis of the distribution network, in the period December 2005–January 2006. Each monitoring point was sampled 1–4 times for this study. The quality control of both systems, with respect to sampling frequency and analytical methodologies, followed the recommendations of the drinking water directive (Council Directive 98/83/EC). Some indicative parameters are presented in Table 1. WWTP isolates were recovered over four sampling campaigns in the period January–December 2005, from raw and treated wastewater of a sewage treatment plant, receiving mainly (~70%) domestic effluents. This WWTP has a preliminary treatment to remove voluminous and settleable solids and a secondary treatment consisting of a biological activated sludge process (Ferreira da Silva et al., 2006, 2007). All the treated water samples examined in this study were conforming to the respective recommended quality standards (Council Directive 91/271/EEC; Council Directive 98/83/EC).

Staphylococci isolation and identification

A total of 19 isolates of the WTP, 175 from the WDN and 48 from the WWTP were examined in this study. Staphylococci were isolated on Mannitol Salt Agar (Pronadisa, Madrid, Spain) at 30 °C, from yellow or red colonies (typical staphylococci colonies as described by Difco Manual, 1984). Additionally, 24 isolates (10 from WTP and 14 from WWTP) were isolated on other media (m-Enterococcus agar, Tergitol-7-agar with triphenyltetrazolium chloride, Bile Esculin agar and *Pseudomonas* Isolation agar). Cultures were purified by sub-culturing on Plate Count agar (5 g L^{-1} tryptone, 2.5 g L^{-1} yeast extract, 1 g L^{-1} dextrose, 15 g L^{-1} agar, PCA, Pronadisa, Madrid, Spain) and preserved at −80 °C in nutritive broth supplemented with 15% (v/v) glycerol. Preliminary characterization included Gram staining, and the tests for cytochrome *c* oxidase, catalase and coagulase (Biokar Diagnostics, Beauvais, France).

The identification of the isolates to the species level, comprehended three stages: 1) the Random Amplified Polymorphic DNA (RAPD) analysis, to establish resemblance groups representative of different species; 2) the analysis and BLAST search of the sequence of the 16S rRNA gene of selected isolates, to infer about the closest phylogenetic neighbours and 3) the further comparative analysis with the type strains of the different species of CNS, to confirm the identification and to define the taxonomic groups composed by closely related species. For DNA extraction, a loop of fresh colonies was suspended in 100 μL of Tris–EDTA (10 mM, pH 8.0): lysostaphin (2 mg mL^{-1}; Sigma-Aldrich, Milwaukee, Wisconsin, USA) (20:1, v/v) and incubated 3 h at 37 °C. Cells lysis and degradation proceeded with SDS (2%), 1 h at 60 °C and proteinase K (0.1 mg mL^{-1}; Q.Biogene, Montreal, Quebec, Canada), 1 h at 37 °C. The DNA was extracted from the cell debris with 100 μL of chloroform: isoamylic alcohol (24:1, v/v). The aqueous phase obtained after 10 min centrifugation at 12000 rpm, was precipitated with 100 μL of cold ethanol. The DNA extract was resuspended in 30 μL of ultra-pure water. The RAPD-PCR genotyping was performed using the primer M13 (5′GAGGGTGGCGGTTCT3′) for all isolates and, additionally, the primers OPA3 (5′AGTCAGCCAC3′) and ERIC1R (5′ATGTAAGCTCCTGGGGATTCAC3′) were used to assess clonal relatedness (Versalovic et al., 1991; Ferreira da Silva et al., 2006, 2007). Amplification reactions were performed in a volume of 25 μL containing: 0.75 U Taq polymerase (Fermentas, Vilnius, Lithuania), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5.0 μM of primer and 0.5 μL of DNA suspension. After 5 min at 94 °C, samples were subjected to 45 cycles of amplification (Biometra, Goettingen, Germany), as follows: 1 min at 94 °C, 1 min at 34 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. Polymorphic DNA fragments were analysed by electrophoresis in a 1.5% agarose gel in Tris-acetate–EDTA buffer (pH8). The RAPD patterns were compared and the resemblance groups were formed as described before (Ferreira da Silva et al., 2006, 2007). The 16S rRNA gene sequence was analyzed in a representative number (10–25%) of isolates of each RAPD group and in all the isolates harbouring unique or poor genotypes. In summary, the gene sequence was examined in the totality of the WWTP isolates, in 60% of the WTP isolates and in 25% of the WDN isolates. The amplicons of 800–1400 bp were obtained as described before (Ferreira da Silva et al., 2007) and used for BLAST search (http://www.ncbi.nlm.nih.gov). Values of 16S rRNA gene sequence similarity higher than 99% were a first indication of the phylogenetic affiliation of the isolates. Subsequently, the comparison of the test sequences with those of the type strains of the different validly named CNS species (MEGA software version 3.1; Kumar et al., 2004) allowed the identification of the isolates to the species level and its organization into species groups as proposed by Takahashi et al. (1999).

Table 1
Physicochemical and microbiological characteristics of the WTP and WDN water.

Parameter	Range (min–max)	
	WTP	WDN
pH (Sorensen scale)	6.4–8.2	7.2–8.5
Alkalinity	21–110	58
Total organic carbon (mg L^{-1} C)	0.8–4.2	0.8–2.7
Conductivity (μS cm^{-1}, at 20 °C)	88–334	196–295
Total hardness (mg L^{-1} $CaCO_3$)	28–143	89–90
Total suspended solids (mg L^{-1})	5.2–25.0	<2.5
Dissolved oxygen (% saturation)	63–98	90 – 148
Chloride (mg L^{-1} Cl)	9.0–20.0^{t}	16.5–19.0
Residual chloride (mg L^{-1} Cl_2)	0.3–1.2	<0.1–1.9
Iron (μg L^{-1} Fe)	20–843	<30–1010
Nitrate (mg L^{-1} NO_3)	1.5–8.4	2.5–7.1
Sodium (mg L^{-1} Na)	7.0–12.0	10.8–14.3
Total heterotrophs (22 °C) (CFU mL^{-1})*	0^{t}–10^{3r}	0–>10^{2}

r, raw water; t, treated water; *Total heterotrophs were enumerated on the medium R2A.

Determination of antibiotic, desinfectant, heavy metal resistance phenotypes

The antimicrobial activity of antibiotics, heavy metal and disinfectant solutions was assayed using the agar diffusion method, as described before (Ferreira da Silva et al., 2006, 2007). Eleven antibiotics, representative of different classes (beta-lactams, tetracycline, sulphamides, macrolides, fluoroquinolones and aminoglycosides) and others to which staphylococci are referred to present high prevalence or emergent resistance, were included in this study. The adopted interpretation criteria based on inhibition zone diameters were as follows (mm): AML (amoxicillin) 25 μg: R<14, I=14–20, S>21; TET (tetracycline) 30 μg: R<17, I=17–18, S>19; SXT (sulfamethoxazole–trimethoprim) 25 μg: R<10, I=10–15, S>16; CIP (ciprofloxacin) 5 μg: R<19, I=19–21, S>22; GEN (gentamicin) 10 μg: R<14, S>15; ERY (erythromycin) 15 μg: R<17, I=17–21, S>22; MET (methicillin) 5 μg: R<15, S>15; OXA (oxacillin) 1 μg: R<14, S>15; CLI (clindamycin) 2 μg: R<15, S>15; PEN (penicillin G) 10 IU: R<8, I=8–28, S>29 and VAN (vancomycin) 5 μg: R<12, S>12. Methicillin, oxacillin and vancomycin susceptibility were assayed on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 2% NaCl and when inhibition zones inferior to 12 or 15 mm were observed, resistance phenotypes were confirmed using the ATB™ STAPH 5 (BioMérieux, Marcy l'Etoile, France), according to the manufacturer instructions. Additionally, the presence of the gene *mec*A was screened by PCR (see below). The phenotype of erythromycin induced clindamycin resistance was tested by the D-test, placing a 2 μg disk of clindamycin 15 mm away from the edge of a 15 μg erythromycin disk, onto Mueller-Hinton agar plates

Table 2
Primers used for antibiotic resistance gene detection.

Target gene	Fragment size (bp)	Primer sequence	$T_{annealing}$ (°C)
*msr*A[a]	940	5′ GGC ACA ATA AGA GTG TTT AAA GG 3′ 5′ AAG TTA TAT CAT GAA TAG ATT GTC CTG TT 3′	50
*erm*A[b]	190	5′ AAG CGG TAA ACC CCG CTG A 3′ 5′ TTC GCA AAT CCC TTC TCA AC 3′	50
*erm*C[b]	299	5′ AAT CGT CAA TTC CTG CAT GT 3′ 5′ TAA TCG TGG AAT ACG GGT TTG 3′	50
*mec*A[b]	314	5′ CCT AGT AAA GCT CCG GAA 3′ 5′ CTA GTC CAT TCG GTC CA 3′	45

[a] Lina et al. (1999).
[b] Ardic et al. (2005).

(O'Sullivan et al., 2006). All the antibiotics used were from Oxoid (Basingstoke, Hampshire, United Kingdom).

The disinfectant and heavy metal solutions tested were hydrogen peroxide (1.5%, w/w) (HP), commercial bleach (<2.5%, w/w sodium hypochloride) (HC) and the salts (Merck, Darmstadt, Germany) 50 mM $Cd(NO_3)_2 4H_2O$, 200 mM $ZnSO_4$, 200 mM $NiCl_2.6H_2O$ and 50 mM $HgCl_2$. The strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* DSM 1104 (=ATCC 25923) were included in each experimental set as controls and presented a variation of inhibition diameters of 1–2 mm. The variation of inhibition diameters (mm) obtained for antibiotics, disinfectants and heavy metals were as follows: AML, 15–58; TET, 0–42; SXT, 0–38; CIP, 21–40; MET, 9–40; OXA, 0–33; GEN, 0–38; ERY, 0–34; CLI, 0–34; PEN, 0–53; VAN, 12–21; HP, 11–42; HC, 15–48; Ni, 10–32; Cd, 13–43; Zn, 11–29; Hg, 12–38. The correlation between the inhibition diameters originated by antibiotics, disinfectants and heavy metals was assessed as described before (Ferreira da Silva et al., 2007), using a Pearson correlation analysis (SPSS 15.0 for windows). According to this analysis a positive correlation was observed when, for a representative group of isolates, the inhibition zone produced by one drug varied (increased or decreased) consistently with that produced by another drug, whereas a negative correlation resulted from the opposite variation of the inhibition halos.

Detection of resistance genetic determinants

The genes *msr*A, *erm*A, *erm*C, and *mec*A were screened using primers described before (Table 2). For the detection of these genes, the PCR mixture was composed of 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μM each primer, 0.5 U Taq polymerase (Fermentas, Vilnius, Lithuania) and 5 μL of DNA for a reaction of 25 μL. The PCR protocol consisted of 10 min of initial denaturation at 94 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at annealing temperature (Table 2), 1 min at 72 °C and a final extension of 10 min at 72 °C. PCR products were analysed by electrophoresis in a 1% agarose gel in Tris-acetate–EDTA buffer (pH 8). A methicillin resistant strain of *Staphylococcus aureus*, which according to PCR and further amplicon sequence analysis possessed the gene *mec*A, was used as positive control for detection of this genetic determinant. For the other genetic determinants under analysis, isolates yielding a positive PCR reaction, confirmed by amplicon sequencing analysis, were defined as positive controls in each PCR run. After settled the positive controls, positive reactions were indicated by comparison with controls and/or through sequencing analysis to confirm the authenticity of the respective PCR product.

Results

Staphylococci diversity

In the WTP total heterotrophic counts were in the range of 10^5 CFU/100 mL in surface water decreasing to 10^3 CFU/100 mL after water treatment. Staphylococci represented a small fraction of these culturable organisms, with counts in the range of 10^2 CFU/100 mL in raw water and 10^0 CFU/100 mL in treated water. In the WDN, heterotrophic counts ranged 10^4–10^2 CFU/100 mL, whereas the staphylococci counts varied over the 33 sampled points between 10^2 and 10^0 CFU/100 mL. Such variations were dependent on the sampling point (area of the WDN) and on the sampling date. For the same sampling site, variable values of staphylococci counts (10^2–10^0 CFU/100 mL) were observed in different dates. A possible,

Table 3
List of CNS examined in this study, organized by site of isolation and taxonomic group and the respective percentages of antibiotic resistance (or intermediary) phenotypes.

Origin	Species	N. of isolates	Species group	PEN	AML	SXT	CIP	TET	GEN	ERY	CLI (c/i)	R2
WTP (*n*=19)	*S. epidermidis*	2^r+4^t	*S. epidermidis* (*n*=10)	(50.0)	(10.0)	0	0	10.0	10.0	50.0	10.0	60.0
	S. capitis	4^r									20.0^i	
	S. pasteuri	4^t	*S. pasteuri* (*n*=8)	(87.5)	(25.0)	0	0	0	(12.5)	25.0	0	50.0
	S. hominis	1^t										
	S. haemolyticus	3^t										
	S. saprophyticus	1^t	*S. saprophyticus* (*n*=1)	0	0	0	0	0	0	0	0	0
WDN (*n*=172)	*S. epidermidis*	49	*S. epidermidis* (*n*=52)	1.9 (42.3)	(3.9)	0	0	1.9	0	17.3	1.9	13.5
	S. capitis	3								(1.9)	5.8^i	
	S. pasteuri	113	*S. pasteuri* (*n*=115)	4.4 (43.5)	(12.2)	0	0	7.8	0	50.4	0.9	29.6
	S. lugdunensis	2									5.3^i	
	S. saprophyticus	4	*S. saprophyticus* (*n*=4)	(25.0)	0	0	0	75.0	0	25.0	25.0^i	25.0
	S. sciuri	1	Other (*n*=1)	(100)	0	0	0	0	0	0	0	0
WWTP (*n*=47)	*S. pasteuri*	5^t	*S. pasteuri* (n=7)	(57.1)	(28.6)	14.3	0	0	(14.3)	0	0	42.8
	S. warneri	1^t										
	S. haemolyticus	1^r										
	S. saprophyticus	1^r+14^t	*S. saprophyticus* (*n*=36)	2.8 (75.0)	0	0	(5.6)	8.3	5.6	16.7	11.1	25.0
	S. xylosus	7^r+6^t									2.8^i	
	S. arletae	1^t										
	S. cohnii	3^r										
	S. equorum	1^t										
	S. succinus	1^r+2^t										
	S. lentus	1^t	Other (*n*=4)	25.0 (25.0)	(50.0)	0	0	25.0	0	50.0	0.0	100
	S. pettenkoferi	1^r									25.0^i	
	S. simulans	2^t										

r, isolates from raw water; t, isolates from treated water.
No resistance was found against methicillin, oxacillin and vancomycin; intermediary resistance phenotypes are indicated between parenthesis; c, constitutive resistance; i, erythromycin induced clindamycin resistance.
R2, resistant or intermediary phenotype to two or more antibiotics.

although speculative, explanation is that the detachment of staphylococci-containing biofilm particles may contribute to a transient increase of culturable cells. In fact, this phenomenon has been described by other authors (e.g. Williams et al., 2004). Even though these data indicate that the WDN may be colonized by CNS it is worth noting that several attempts to isolate staphylococci on Mannitol Salt agar from taps water were unsuccessful. Wastewater contained, as expected, the higher counts of total heterotrophic bacteria and staphylococci. Total heterotrophic counts were in the range of 10^8 CFU/100 mL in raw influent, being 10 times lower in the treated effluent, and the staphylococci were, respectively, in the order of magnitude of 10^5–10^3 CFU/100 mL (Ferreira da Silva et al., 2006, 2007).

A total of 242 staphylococci isolated from WTP (19), WDN (175) and WWTP (48) were identified to the species level (Table 3). Among these isolates only four, three from the WDN and one from the treated effluent of the WWTP, were coagulase positive and identified through the 16S rRNA gene sequence analysis as *Staphylococcus aureus* (data not shown). These four isolates were not further analysed.

Through the RAPD analysis, most of the WDN CNS (150) was divided into three genotypic groups, identified, respectively, as *S. saprophyticus*, *S. pasteuri* and *S. epidermidis*, which included a subgroup of three *S. capitis*. Twenty two isolates of the WDN could not be integrated in any group, as presented poor or unique profiles, and were further identified as *S. pasteuri*, *S. epidermidis*, *S. lugdunensis* and *S. sciuri*. The WWTP isolates were divided into two major RAPD groups, corresponding respectively to *S. saprophyticus* and *S. xylosus*. Other groups represented by less than ten isolates and ungrouped profiles corresponded to different species (Table 3). The WTP isolates originated six RAPD patterns, corresponding to different species. Table 3 lists the identification of the isolates per site of isolation and organizes the species into phylogenetic groups as proposed by Takahashi et al. (1999).

In general, different patterns of CNS were observed in wastewater and in water destined to human consumption. In wastewater, *S. saprophyticus* and *S. xylosus* were the prevalent species of culturable CNS, whereas in the WDN the predominant culturable species were *S. epidermidis* and *S. pasteuri*. These two species and S. *capitis* were also cultured from the WTP, and presumably at least *S. capitis* and *S. pasteuri* from WTP and WDN were clonally related, as the comparative analysis of the RAPD genotypes (using the primers M13, OPA3 and ERICR1) revealed the occurrence of similar patterns (Fig. 1). Such observation suggests that CNS may resist water disinfection and, even if they reach values below the detection limit, may colonize the water distribution structures. In spite of this, in the WDN the diversity of CNS was considerably lower than in the WTP and WWTP, even thought a higher number of isolates was analysed.

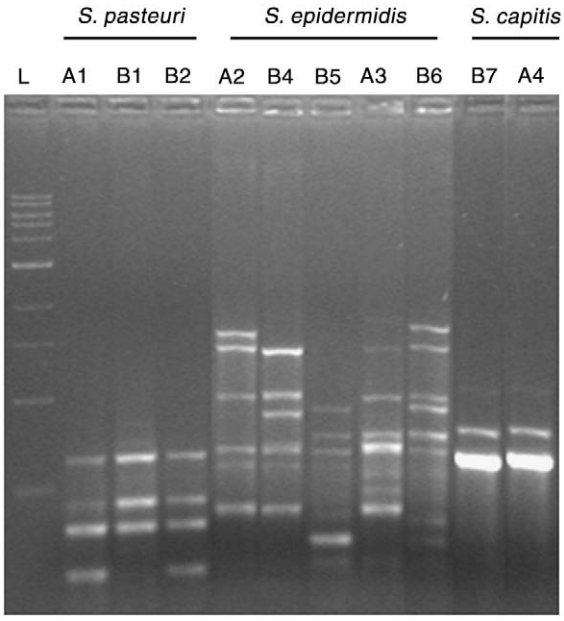

Fig. 1. RAPD typing with primer M13 of some isolates belonging to the two predominant groups found in WDN. L, DNA ladder; A–WTP isolates; B–WDN isolates.

Resistance phenotypes

In order to compare the antimicrobial resistance patterns observed in the CNS isolated from the different types of water, antibiotic susceptibility was determined for 11 antibiotics (Table 3). According to the criteria used, no resistant strains were found for the antibiotics methicillin (as confirmed by the absence of the gene *mec*A), oxacillin and vancomycin, and only intermediary resistance phenotypes were observed for ciprofloxacin. Reduced susceptibility (intermediary or resistance phenotype) to sulfamethoxazole/trimethoprim and gentamycin was also rare, observed, respectively in one WWTP and in four WTP isolates (Table 3). Intermediary resistance phenotypes were frequently observed for the beta-lactam antibiotics penicillin and amoxicillin, in all species groups and in the three types of water (Table 3). Among the 126 isolates with reduced susceptibility (resistance or intermediary phenotype) to penicillin, only 20 (2 WTP, 13 WDN, 5 WWTP) presented the same phenotype to amoxicillin, hinting a hypothetic common mechanism of antibiotic tolerance. Although tetracycline resistance was observed in only 18 isolates, it was detected in all types of water (1 WTP, 13 WDN, 4 WWTP) and was distributed by all species groups. The most widespread and frequent resistance phenotype was observed for the macrolide erythromycin, detected in 84 isolates of all types of water and species groups. The data obtained suggest that this phenotype may be more frequent in the *S. pasteuri* and *S. epidermidis* species groups, explaining the high rates of resistance to this antibiotic in the WDN isolates, where those organisms were prevalent. In fact, this resistance phenotype, highly represented in the WDN, was regularly observed over the sampling period, hinting a possible resident colonization. In the WWTP, erythromycin resistance was mainly found in isolates of the raw affluent (7 resistant out of 14, against 1 out of 33 in the treated effluent). This result may suggest an efficient elimination of erythromycin resistant staphylococci during wastewater treatment. However a much higher number of isolates would be necessary to reach valid conclusions. Although erythromycin and clindamycin resistance are frequently referred as being associated phenotypes, this was not observed in the present study. Of the 84 isolates resistant to erythromycin only 3 had simultaneously reduced susceptibility to clindamycin (1 from the WTP and 2 from the WDN). The occurrence of resistance or intermediary phenotypes to two or more antibiotics (Table 3) presented high prevalence values, mainly in untreated water, with 52.6% for WTP, 25.0% for WDN and 34.0% for the WWTP isolates. Some of these included erythromycin and tetracycline or gentamycin resistance. But, in most of the cases, erythromycin resistance was combined with penicillin intermediary phenotype, although never with penicillin or amoxicillin resistance.

Resistance genetic determinants

Given the high frequency of erythromycin resistance phenotype observed mainly in staphylococci isolated from the WDN, the genetic determinants responsible for such a phenotype were also investigated. The genetic determinants screened were the *msr*A genes, encoding an efflux pump, and the genes *erm*A and *erm*C, both associated with the production of a 23S rRNA methylase, that may trigger a cross resistance to lincosamides (Table 4) (Leclercq and Courvalin, 1991). These three determinants explained all the erythromycin resistance phenotypes observed with the prevalence of the

gene *msr*A (found in 70 out of 238 isolates), followed by *erm*C (detected in 13 out of 238 isolates). The gene *msr*A was normally associated with erythromycin resistance whereas *ermC* was observed in erythromycin resistant isolates with erythromycin induced clindamycin resistance. In the WDN, 62 out of 73 of the isolates that harboured any of these three genes encoded the efflux pump. In most of the isolates encoding the efflux pump *msr*A, positive PCR reactions were observed using both primers pairs reported by Lina et al. (1999). In contrast, in three isolates from raw wastewater identified as *S. xylosus*, *S. cohnii* and *S. succinus* only one of those primers sets (msrA, according to Lina et al. (1999) nomenclature) gave a positive PCR reaction. The simultaneous presence of *msr*A and *erm*C was also exceptional, observed in three *S. pasteuri* from the WDN and in one *S. succinus* from the WWTP. The gene *erm*A, although rarely found, was observed in isolates from the three types of water and its occurrence was associated with *S. epidermidis* and *S. simulans* (Table 4).

Correlation analysis on antibiotics, heavy metals and desinfectants susceptibility

Although resistance phenotype determination is of surmount importance for clinical isolates, the tolerance to antimicrobial substances, even when these are below the resistance/susceptibility breakpoints, may represent a selective advantage for the organism in the environment. It has been hypothesized that in the environment, bacteria may face different types of chemical aggressions, capable of selecting positively or negatively antibiotic tolerance. Presumably, bacteria thriving in wastewater, where antibiotic residues or heavy metals may be discharged, face different challenges than those surviving in a water supplying system, where the organic content and chemical contamination is absent or at trace levels. Such environmental pressures may also be responsible for the prevalence of specific groups of organisms able to deal with the environmental conditions imposed. A Pearson correlation analysis between the inhibition diameters produced by antibiotics, heavy metals and disinfectants was made in order to assess whether the susceptibility to two substances could be related (Fig. 2). This analysis involved the major taxonomic groups — S. epidermidis and S. pasteuri isolated from the WDN and S. saprophyticus from the WWTP. The disinfectants tested were bleach and hydrogen peroxide, which have active principles and mechanisms of action similar, respectively, to chlorination and ozonization, used in water disinfection. One of the hypotheses behind the assay of these agents was that water disinfection might eliminate or select for antibiotic resistant CNS. However, given that no significant correlations were found between these disinfectants and antibiotics, it is suggested that such active principles may not influence antibiotic resistance elimination or selection. For the other antimicrobial agents, with a few exceptions, a different pattern of significant correlations was observed for the wastewater S. saprophyticus and for the water distribution system S. pasteuri groups. In this last group were observed consistently significant positive correlations between Hg and beta-lactams. This fact may indicate a similar behaviour in the presence of both types of antimicrobials, meaning that high susceptibility or tolerance to both agents may occur simultaneously in these isolates. In contrast, the wastewater isolates of S. saprophyticus group evidence consistently positive correlations between beta-lactams and antibiotics of other classes, as tetracycline, ciprofloxacin, gentamicin or sulfamethoxazole/trimethoprim. It is interesting to note, that the same pattern of association was observed before for *Escherichia coli* isolates recovered from the same wastewater treatment facility (Ferreira da Silva et al., 2007). An interesting finding was that erythromycin, to which about 30% of the isolates were resistant, was negatively correlated, in S. pasteuri and in S. saprophyticus groups, with the peptidoglycan targeting antibiotics examined (amoxicillin, penicillin, vancomycin, methicillin and oxacillin). This finding confirms the fact that none of the erythromycin resistant isolates was also resistant to penicillin, although the occurrence of an intermediary phenotype was observed in some cases. This observation suggests that the acquisition of resistance to erythromycin and peptidoglycan targeting antibiotics may result from distinct physiological events. Moreover, erythromycin resistance was not associated with the reduced susceptibility to clindamycin. This observation finds support on the susceptibility patterns reported above and on the genetic determinants detected in these isolates (Table 4). In fact, the gene *msr*A is normally associated with macrolide but not with lincosamide resistance (Lina et al., 1999).

Table 4
Distribution of genes related with macrolide resistance in CNS isolated from different types of water.

Origin	Species	Gene (*n*)	Resistance phenotype (*n*)
WTP	*S. epidermidis*	*erm*A	ERY, CLI
		*erm*C (2)	ERY, CLI^{i}
			TET, ERY, CLI^{i}
		*msr*A (2)	ERY
			GEN, ERY
	S. haemolyticus	*msr*A (2)	ERY
			PEN*, AML*, ERY
	S. saprophyticus	*msr*A	none
WDN	*S. epidermidis*	*erm*A	PEN*
		*erm*C (3)	ERY, CLI^{i}
			ERY, CLI^{i}
			TET, ERY, CLI^{i}
		*msr*A (7)	ERY (3)
			PEN*, ERY (3)
			ERY*, CLI
	S. pasteuri	*ermC (6)*	TET
			TET, ERY, CLI^{i}(2)
			PEN*, ERY, CLI^{i}
			PEN*, AML*
			ERY, CLI^{i}
		*msr*A (51)	ERY (35)
			ERY, CLI^{i}
			PEN*, ERY (11)
			AML*, ERY
			PEN*, AML*, ERY (3)
		*erm*C + *msr*A (3)	ERY
			ERY, CLI
			PEN*, TET, ERY, CLI^{i}
	S. saprophyticus	*erm*C	TET, ERY, CLI^{i}
		*msr*A	TET
WWTP	*S. cohnii*	*msr*A	PEN*, ERY
		*msr*A (2)	ERY (2)
	S. succinus	*erm*C + *msr*A	PEN*, ERY, CLI^{i}
	S. simulans	*erm*A	PEN*, AML*, ERY, CLI^{i}
	S. pettenkoferi	*msr*A	ERY
	S. xylosus	*erm*C	none
		msrA	PEN*, TET, ERY
		msrA	ERY*

n, indicates respectively, the number of isolates with that genotype or phenotype; *, intermediary phenotype; ^{i} induced resistance phenotype.

Discussion

Water is one of the most relevant vehicles of bacterial propagation and dissemination. The presence of antibiotic resistant bacteria or resistance determinants in drinking water has been reported sporadically for more than 20 years in different countries (Armstrong et al., 1981; Pavlov et al., 2004; Schwartz et al., 2004; Ram et al., 2008). Human activities have led to the establishment of a new water cycle, where wastewater treatment and water catchment/treatment/distribution represent two key stages. Throughout this cycle, bacteria tolerant to antibiotics may never be eliminated. Staphylococci are ubiquitous bacteria with widespread distribution, and their presence in different types of water has been reported (Armstrong et al., 1981; Kessie et al., 1998; Harakeh et al., 2006; Papadopoulou et al., 2008). The present study aimed at assessing the distribution of staphylococci and their resistance trends in different types of water. While in WWTP

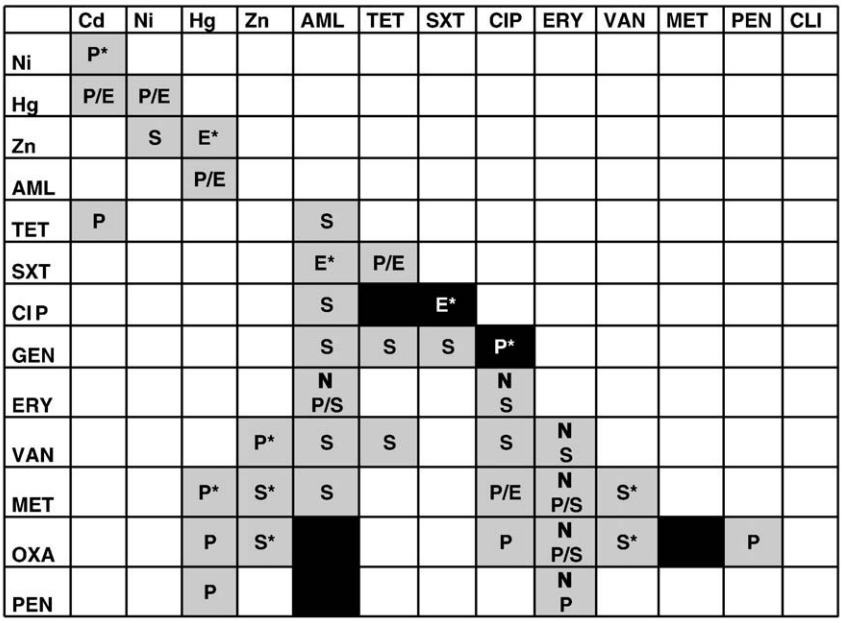

Fig. 2. Pearson correlation for the inhibition zones originated by different antimicrobial agents. It was considered a significant level <0.005, except when indicated by * where a significant level <0.01 was observed. Gray shadowing, indicates a significant correlation observed for members of *S. pasteuri* group (P), *S. epidermidis* group (E), or *S. saprophyticus* group (S); Black shadowing indicates significant correlations found for the three species groups: N, indicates significant negative correlation. No significant correlations were found between bleach or hydrogen peroxide and the antibiotics tested.

and in the WTP raw water the presence of CNS was expected, their occurrence in the WDN may represent an undesired colonization, although fully explained by the remarkable ubiquity of staphylococci. In the WDN, CNS were clearly minor components of the whole bacterial community, as could be inferred from the total heterotrophic counts. The presumable origins of colonization by CNS in the WDN were not the focus of this study and only hypothesis can be proposed — for instance, that CNS are not completely eliminated during water treatment, allowing their entrance into the distribution system or that undetectable micro-fissures in the distribution network may allow the intrusion of such organisms. The first hypothesis cannot be excluded, at least for some organisms, as similar genotypes were found in the WTP and in WDN. However, both origins of colonization may justify the occurrence of CNS in the WDN. In this respect it is worthy of note that no recommendations about the screening of staphylococci are established for drinking water in European legislation (Council Directive 98/83/EC), which makes their presence unnoticeable for quality control entities. Moreover, routine analysis made to tap water may also mask the presence of such organisms in the water distribution system. As was confirmed during this study, several attempts to isolate staphylococci from tap water were unsuccessful.

In this study, different species of staphylococci were observed to prevail in each type of water examined. Nevertheless, phenotypes of reduced susceptibility to some antibiotics were detected in isolates from all types of water and some of them, irrespective of the species group or source, presented resistance or intermediary phenotype to at least two antibiotics, frequently belonging to different families (Table 3). Erythromycin resistance was observed to be predominant among the isolates examined, independently of their origin. High rates of erythromycin resistance are reported for CNS isolates of animal or human-clinical origin (Lina et al., 1999; Nawaz et al., 2000; Novotna et al., 2005; Lüthje and Schwartz, 2007), thus its presence and presumable persistence in water for human consumption may be worrisome.

In the present study it was observed that erythromycin resistance is probably mainly due to drug efflux, as the gene *msr*A was detected in the majority of the isolates. The same genetic determinants were reported as highly frequent not only in CNS isolated from food products as cheese and raw meat (Perreten et al., 1998), but also in *S. aureus* and CNS of clinical origin and in distantly related bacteria (Lina et al., 1999; Nawaz et al., 2000; Novotna et al., 2005; Ojo et al., 2006). This reinforces the dynamic of antibiotic resistance genetic determinants and suggests that no species-specific or environmental barrier halts the passage of these genes from and into clinical environments and clinically relevant bacteria.

The occurrence of antibiotic tolerant bacteria in water for human consumption poses a risk for which poor or no assessment is available (Bartram et al., 2003). One of the aspects of concern to assess the risks associated with its presence is the prediction of their behaviour in the presence of other stress factors, namely antimicrobial substances. The correlation analysis made in this study demonstrated that CNS isolated from wastewater behaved differently from those recovered from water destined to human consumption, hinting different patterns of response to environmental stresses. These observations may explain also the prevalence of distinct species in both types of water. Further studies on the persistence of bacteria over the water cycle as well as on the effect of potential selective pressures on the dissemination or eradication of antibiotic tolerant bacteria may represent a valuable contribute for risk assessment studies and antibiotic resistance control.

Conlusions

- This study demonstrates that CNS may colonize water destined to human consumption, even when the quality standards for drinking water are fulfilled.
- Erythromycin resistance was highly prevalent in the CNS isolates examined and most of these isolates presented the gene *msr*A, encoding an efflux pump for this antibiotic; the same determinant has been reported in bacteria belonging to different taxonomic groups isolated from different origins, including clinical.
- The majority of CNS isolated from the drinking water distribution network belonged to the species *S. pasteuri*, which has widespread distribution in food and in the environment, and may represent a relevant antibiotic resistance reservoir, mainly in habitats with restrictive conditions and reduced staphylococcal diversity.

– The occurrence of antibiotic resistant CNS in water destined to human consumption has never been addressed before and may represent a hazard under conditions capable of favouring their overgrowth, e.g. biofilm formation or antibiotherapy.

Acknowledgements

Authors gratefully acknowledge the senior scientists of the WTP, WDN and WWTP for their support and helpful discussions, and Profs. Gerard Lina (Centre National de Référence des Toxémies Staphylococciques, Faculté de Médecine, Lyon) and Manuela Pintado and Freni Tavaria (CBQF-UCP-ESB) for strains used as controls. This study was financed by Fundação Calouste Gulbenkian and by Fundação para a Ciência e a Tecnologia (project PTDC/AMB/70825/2006 and grant SFRH/BD/27978/2006).

References

Ardic N, Ozyurt M, Sareyyupoglu B, Haznedaroglu T. Investigation of erythromycin and tetracycline resistance genes in methicillin-resistant staphylococci. Int J Antimicrob Agents 2005;26:213–8.

Armstrong JL, Shigeno DS, Calomiris JJ, Seidler RJ. Antibiotic resistant bacteria in drinking water. Appl Environ Microbiol 1981;42:277–83.

Bartram J, Cotruvo J, Exner M, Fricker C, Glasmacher A, editors. Heterotrophic plate counts and drinking-water safety: the significance of HPCs for water quality and human health. London: IWA Publishing; 2003.

Council Directive 91/271/EEC, of 21 May 1991 concerning urban waste-water treatment.

Council Directive 98/83/EC, of 3 November 1998 on the quality of water intended for human consumption as amended by regulation 1882/2003/EC.

Difco Manual: dehydrated culture media and reagents for microbiology. Difco, 10th ed., Detroit, Michigan: Difco laboratories 1984.

Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, et al. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol 2008;8:43.

Eichler S, Christen R, Höltje C, Westphal P, Bötel J, Brettar I, et al. Composition and dynamics of bacterial communities of a drinking water supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting. Appl Environ Microbiol 2006;72:1858–72.

Ferreira da Silva M, Tiago I, Veríssimo A, Boaventura AR, Nunes OC, Manaia CM. Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. FEMS Microbiol Ecol 2006;55:322–9.

Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant. FEMS Microbiol Ecol 2007;60:166–76.

Harakeh S, Yassine H, Hajjar S, El-Fadel M. Isolates of *Staphylococcus aureus* and *saprophyticus* resistant to antimicrobials isolated from the Lebanese aquatic environment. Mar Pollut Bull 2006;52:912–9.

Huebner J, Goldmann DA. Coagulase-negative staphylococci: role as pathogens. Annu Rev Med 1999;50:223–36.

Iacumin L, Comi G, Cantoni C, Cocolin L. Ecology and dynamics of coagulase-negative cocci from naturally fermented Italian sausages. Syst Appl Microbiol 2006;29:480–6.

Irlinger F. Safety assessment of dairy microorganisms: Coagulase-negative staphylococci. Int J Food Microbiol 2008;126:302–10.

John JF, Harvin AM. History and evolution of antibiotic resistance in coagulase-negative staphylococci: susceptibility profiles of new anti-staphylococcal agents. Ther Clin Risk Manag 2007;3:1143–52.

Kessie G, Ettayebi M, Haddad AM, Shibl AM, Al-Shammary FJ, Tawfik AF, et al. Plasmid profile and antibiotic resistance in coagulase-negative staphylococci isolated from polluted water. J Appl Microbiol 1998;84:417–22.

Kloos WE, Schleifer KH, Götz F. The genus *Staphylococcus*. . chapter 63, vol. 2In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH, editors. The Prokaryotes. 2nd ed. New York: Springer; 1992. p. 1369–420.

Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–63.

Leclercq R, Courvalin P. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother 1991;35:1267–72.

Lina G, Quaglia A, Reverdy M, Leclercq R, Vandenesch F, Etienne J. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob Agents Chemother 1999;43:1062–6.

Lüthje P, Schwartz S. Molecular basis of resistance to macrolides and lincosamides among staphylococci and streptococci from various animal sources collected in the resistance monitoring program BfT-GermVet. Int J Antimicrob Agents 2007;29:528–35.

Nawaz MS, Khan SA, Khan AA, Khambaty FM, Cerniglia CE. Comparative molecular analysis of erythromycin-resistance determinants in staphylococcal isolates of poultry and human origin. Mol Cell Probes 2000;14:311–9.

Norton DC, LeChevallier WM. A pilot study of bacteriological population changes through potable water treatment and distribution. Appl Environ Microbiol 2000;66:268–76.

Novotna G, Adamkova V, Janata J, Melter O, Spizek J. Prevalence of resistance mechanisms against macrolides and lincosamides in methicillin-resistant coagulase-negative staphylococci in the Czech Republic and occurrence of an undefined mechanism of resistance to lincosamides. Antimicrob Agents Chemother 2005;49:3586–9.

Ojo KK, Striplin MJ, Ulep CC, Close NS, Zittle J, Luis H, et al. *Staphylococcus* efflux *msr*(A) gene characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* isolates. Antimicrob Agents Chemother 2006;50:1089–91.

O'Sullivan MVN, Cai Y, Kong F, Zeng X, Gilbert GL. Influence of disk separation distance on accuracy of the disk approximation test for detection of inducible clindamycin resistance in *Staphylococcus* spp. J Clin Microbiol 2006;44:4072–6.

Papadopoulou C, Economou V, Sakkas H, Gousia P, Giannakopoulos X, Dontorou C, et al. Microbiological quality of indoor and outdoor swimming pools in Greece: investigation of the antibiotic resistance of the bacterial isolates. Int J Hyg Environ Health 2008;211:385–97.

Pavlov D, Wet CME, Grabow WOK, Ehlers MM. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. Int J Food Microbiol 2004;92:275–87.

Perreten V, Giampà N, Schuler-Schmid U, Teuber M. Antibiotic resistance genes in coagulase-negative staphylococci isolated from food. Syst Appl Microbiol 1998;21:113–20.

Ram S, Vajpayee P, Shanker R. Contamination of potable water distribution systems by multiantimicrobial-resistant enterohemorrhagic *Escherichia coli*. Environ Health Perspect 2008;116:448–52.

Rupp ME, Archer GL. Coagulase-negative staphylococci: pathogens associated with medical progress. Clin Infect Dis 1994;19:231–43.

Schwartz T, Kohnen W, Jansen B, Obst U. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol Ecol 2004;43:325–35.

Smith AJ, Jackson MS, Bagg J. The ecology of *Staphylococcus* species in the oral cavity. J Med Microbiol 2001;50:940–6.

Takahashi T, Satoh I, Kikuchi N. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. Int J Syst Bacteriol 1999;49:725–8.

Versalovic K, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 1991;19:6823–31.

Williams MM, Domingo JWS, Meckes MC, Kelty CA, Rochon HS. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J Appl Microbiol 2004;96:954–64.