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Zuzana Čuvalová, Monika Pipová*, Miriam Kantíková, Andrea Brtková, Jozef Fajber

Virulence factors and antimicrobial resistance of coagulase-negative staphylococci isolated from drinking water

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Abstract: Little has been published about the occurrence, speciesidentification,andpathogenicpotentialofcoagulase-negative staphylococci (CoNS) present in drinking water. In this study, ten species were identified among 57 isolates of staphylococci from 756 samples of chlorinated drinking water taken from public distribution networks in the Slovak Republic. *S. warneri* (37%), *S. haemolyticus* (23%), and *S. saprophyticus* ssp. *saprophyticus* (14%) were identified most frequently. Isolates did not produce coagulase, DNase, or hyaluronidase; production of gelatinase and lecithinase was observed in 28 and 22 isolates, respectively. Genetically encoded ability for production of enterotoxin SED was revealed in two isolates. Among ten antibiotics tested, resistance to ampicillin (66.7%), penicillin (64.9%), and erythromycin (57.9%) were observed most frequently. Resistance to gentamicin, vancomycin, or clindamycin was not confirmed. Production of β-lactamase was observed in 64.9% of isolates. Fourty-two isolates were resistant to two or more antibiotics tested, and eight isolates showed multiresistance. The presence of *mec*A gene was confirmed in 8 isolates, while PBP2a was revealed in 7. Two isolates of *S. epidermidis* were identified as methicillin-resistant (MRSE). The results demonstrate that CoNS in chlorinated drinking water may possess virulence factors and show resistance to various antibiotics. Therefore, their pathogenic potential should not be ignored.

Keywords: coagulase-negative staphylococci, drinking water, resistance, *mec*A gene, MRSE, enterotoxins

***Corresponding authors: Monika Pipová,** Department of Food Hygiene and Technology, University of Veterinary Medicine and Pharmacy, 041 81 Košice, Slovak Republic, E-mail: pipova@uvlf.sk
Zuzana Čuvalová, Miriam Kantíková, Andrea Brtková, Jozef Fajber, State Veterinary and Food Institute, 026 01 Dolný Kubín, Slovak Republic

1 Introduction

Staphylococci are ubiquitous Gram-positive and non-motile bacteria that colonize mucous membranes of warm-blooded animals and humans [1,2], and can be divided into two major groups according to their ability to coagulate rabbit plasma: coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS). The cell structure of staphylococci makes them highly tolerant to drying, dehydration and low water activity, which explains, in part, their widespread distribution and persistence in the environment [3]. The presence of staphylococci has been documented in air, soil, water, humans and a variety of animals, as well as in processed food products such as cheeses or fermented sausages [3-7].

In the past, CoNS have been regarded to be non-pathogenic as most of these species can establish a commensal relationship with humans and animals [8,9], but this opinion is changing. The few reports of infections published by clinicians and microbiologists before the 1970s considered CoNS to be contaminants of clinical samples [10]. However, there is now increasing evidence that some species of CoNS (e.g. *S. capitis, S. cohnii, S. epidermidis, S. haemolyticus*, *S. hyicus* ssp. *hyicus* and *S. xylosus* [11-14]) can cause human diseases and nosocomial infections and/or may endanger human health through enterotoxin production [15]. In veterinary medicine, some CoNS species (*S. chromogenes*, *S. simulans* and *S. xylosus*) are also known to be causative agents of infectious mastitis [16,17]. The pathogenic potential of CoNS indicates that their occurrence could represent more significant safety hazards than previously thought in both the clinical environment and in food [1,18]. It is possible that the occurrence of CoNS in drinking water supplies could also represent a significant safety concern.

The aim of this study was to identify species of CoNS isolated from chlorinated drinking water distributed through public water supply network in the Slovak Republic, to reveal the presence of virulence factors, to

test the susceptibility of isolates to some antibiotics and to detect genes encoding resistance to methicillin (*mec*A gene) and the production of enterotoxins. The intensive use of antibiotics in agriculture and both human and veterinary medicines results in their permanent release into the environment [19-21], as well as in the development of antibiotic resistant bacteria [22]. As drinking water is produced from surface water, it represents a way of dissemination of antibiotic-resistant organisms among human and animal populations but also the route which enables resistance genes to become introduced into natural bacterial ecosystems. In such systems, nonpathogenic bacteria could serve as a reservoir of resistance genes [23]. CoNS may also carry genes encoding resistance to various antibiotics [24]. Currently, a particularly serious problem is the spread of multidrug resistance through methicillin-resistant *S. epidermidis* [25,26].

2 Experimental Procedures

2.1 Sampling

Sampling was performed by the trained persons in households, refectories, food-processing establishments, as well as on animal farms throughout the Slovak Republic. Water samples in volumes of 1L were taken aseptically into sterile bottles, and transported to the laboratory for immediate microbiological examination.

2.2 Determination of free chlorine content

Routine control of free chlorine in water was performed during sampling using the Spectroquant Colorimeter Picco (Merck, Germany) in accordance with the requirements of STN EN ISO 7393-2 [27] to check whether the free chlorine reached levels required for public water supply in the Slovak Republic.

2.3 Isolation of staphylococci

Staphylococci were isolated from 756 samples of chlorinated drinking water according to STN EN ISO 8199 [28]. 100 mL of each water sample were filtered through the membrane with 0.45 μm pore size. After filtration, the membrane was aseptically placed onto the dried surface of Baird Parker agar medium (Oxoid, UK), with the membrane/agar surface uppermost, taking care to avoid trapping air bubbles beneath the membrane. Plates with membranes were incubated at 37 ± 2°C for 44 ± 4 h to obtain viable cultures of staphylococcci. Characteristic black colonies were further subcultured onto the dried surface of Columbia blood agar (Oxoid, UK) and incubated at 37 ± 2°C for 24 ± 4 h prior to species identification.

2.4 Species identification

2.4.1 MALDI-TOF mass spectrometry

Isolates of staphylococci were identified with the help of a MALDI BioTyper™ system based on 'protein fingerprints' measured by MALDI-TOF mass spectrometry (Bruker Daltonics, USA). Species achieving MALDI-TOF score values above 2.000 (i.e. highly probable or probable species identification) were further confirmed by phenotypic methods. Isolates of *S. epidermidis* were submitted to species-specific PCR.

2.4.2 Phenotypic confirmation

Species of staphylococci were identified using the STAPHYtest 24 completed with VP-, PYRA- and OXI-tests, as well as with the disk diffusion test for bacitracin (0.04 UI) and novobiocin (5 μg). All the tests were evaluated using the TNW Pro 7.5 identification program (Erba-Lachema, Czech Republic).

2.4.3 Species identification of *S. epidermidis*

Isolates of *S. epidermidis* were also confirmed using the species-specific PCR method detecting the amplicon of 124 bp [29]. Reference strain *S. epidermidis* CCM 4616 (Czech Collection of Microorganisms Brno, Czech Republic) was used as a positive control. Genomic DNA was extracted according to Chomczynski et al. [30]. Quantity and purity of the extracted DNA was evaluated with the help of a NanoVue spectrophotometer (GE Healthcare Life Sciences, UK) at 260 and 280 nm as described by Sambrook et al. [31]. The PCR mixture was composed of 2.5 μl PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.8 μM each primer, 0.5 U *Taq* DNA polymerase (Qiagen, Germany) and 200 ng of genomic DNA for a reaction of 25 μl. Primers (Generi Biotech, Czech Republic) Sep-f (5´–ATCAAAAAGTTGGCGAACCTTTTCA) and Sep-r (5´– CAAAAGAGCGTGGAGAAAAGTATCA), typical for *S. epidermidis*, were used for PCR. The PCR protocol included 15 min of initial denaturation at 94°C, followed by 30 cycles (45 s of denaturation at 94°C, 30 s of annealing at 55°C and 60 s of polymerization at 72°C). Final extension was performed at 72°C within 10 min. Products were analyzed by electrophoresis in a 2% agarose gel

(Bioline, UK), stained with ethidium bromide (10 mg mL^{-1}), viewed by UV transillumination and compared with the standard Marker C (SBS Genetech, China).

2.5 Determination of virulence factors

All the 57 CoNS isolates were tested for the presence of virulence factors (i.e. production of coagulase, DNase, lecithinase, gelatinase and hyaluronidase) associated with their pathogenicity. Coagulase is responsible for clotting of the blood plasma, DNase catalyses degradation of DNA. Both gelatinase and hyaluronidase allow staphylococci to hydrolyse proteins, lecithinase catalyses degradation of lipids.

2.5.1 Coagulase activity

Individual isolates of staphylococci were inoculated into test tubes containing 2 mL of Brain Heart Infusion Broth (Oxoid, UK) and incubated for 18-24 h at 37 ± 1°C. A volume of 0.1 mL of each bacterial culture was then added to 1 mL of reconstituted freeze-dried rabbit plasma (Stafylo PK, Imuna Pharm, Slovakia) to test whether each culture contained coagulase-positive staphylococci (CoPS) or coagulase-negative staphylococci (CoNS). Clotting of the plasma was checked after 1, 2, 4, 6, and 24 h of incubation at 37 ± 1°C.

2.5.2 DNase activity

Isolates of staphylococci were inoculated on the dried surface of DNase agar (Oxoid, UK) and incubated at 37 ± 1°C for 24 h. After incubation, the medium was flooded with 1 N hydrochloric acid. DNA contained in the medium precipitated in the presence of HCl, the medium became turbid and clear zones appeared around DNase-positive strains.

2.5.3 Lecithinase activity

Production of lecithinase was evaluated according to the appearance of individual isolates on the surface of Baird-Parker agar medium containing egg yolk emulsion (Oxoid, UK). Formation of precipitation zones around typical black colonies after a 48-h-incubation at 37 ± 1°C confirmed bacterial lecithinase activity.

2.5.4 Gelatinase activity

The overnight culture of each isolate was inoculated on the dried surface of Brain Heart Infusion Agar (Oxoid, UK) with 0.4% of gelatin added. After incubation for 24 h at 37 ± 1°C the medium was flooded with 15% $HgCl_2$ diluted in 20% HCl. Production of gelatinase was manifested by the formation of clear zones around gelatinase-positive colonies.

2.5.5 Hyaluronidase activity

The decapsulation test was used for the detection of bacterial hyaluronidase production. Columbia blood agar plates (Oxoid, UK) were first inoculated with the reference strain *Streptococcus equi* ssp. *zooepidemicus* CCM 7316 (Czech Collection of Microorganisms, Czech Republic). Isolates of stafylococci tested were then inoculated perpendicularly to a 1.5 cm wide strip of the reference strain. The test was evaluated after a 18-h-incubation at 37 ± 1°C. Decapsulation of *Str. equi* ssp. *zooepidemicus* was observed as a visible hemispherical reduction of mucousal growth in the presence of hyaluronidase-positive isolates.

2.5.6 Detection of genes encoding production of enterotoxins

The presence of genes encoding production of staphylococcal enterotoxins SEA-SEE (*sea*, *seb*, *sec*, *sed* and *see*) was detected with the help of a multiplex PCR method [32]. A 50 µL reaction mixture was created containing 1 µL genomic DNA, 10 mmol L^{-1} Tris-HCl (pH 8.8), 3 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} dNTP, 12.5 pmol L^{-1} of each primer, and 1U *Taq* DNA polymerase (Ecoli s.r.o., Slovakia). Primers are specified in Table 1. The PCR protocol included the following three steps: (1) initial denaturation at 94°C for 2 min, (2) 25 cycles, each consisting of denaturation at 94°C 30 s, annealing at 45°C for 30 s, and extension at 72°C for 30 s, and (3) final extension at 72°C for 2 min. The following reference strains were used as positive controls: *Staphylococcus aureus* (Czech Collection of Microorganisms Brno, Czech Republic): CCM 5756 (gene *sea*), CCM 5757 (gene *seb*), CCM 5984 (gene *sec*), CCM 5973 (gene *sed*), and CCM 5972 (gene *see*). PCR products were separated in a 2% agarose gel, stained with Goldview Nucleic acid stain (Beijing SBS Genetech Co. Ltd, China) and visualized using the DNR Bio Imaging sytem (MiniBIS Pro, Israel).

Table 1 Primers and amplicons used for the detection of *sea-see* genes responsible for the production of staphylococcal enterotoxins SEA-SEE [32].

Primer name	Description	Nucleotide sequence 5'-3'	PCR product size (bp)
SA-U	Universal forward primer	TGTATGTATGGAGGTGTAAC	–
SA-A	Reverse primer for *sea*	ATTAACCGAAGGTTCTGT	270
SA-B	Reverse primer for *seb*	ATAGTGACGAGTTAGGTA	165
SA-C	Reverse primer for *sec*	AAGTACATTTTGTAAGTTCC	102
SA-D	Reverse primer for *sed*	TTCGGGAAAATCACCCTTAA	306
SA-E	Reverse primer for *see*	GCCAAAGCTGTCTGAG	213

2.6 Determination of antimicrobial resistance

Resistance of staphylococcal isolates to 10 antibiotics was determined by the disk diffusion susceptibility test according to Kirby-Bauer [33]. Bacterial suspensions adjusted to the 0.5 McFarland turbidity standard were prepared from overnight cultures on the Columbia blood agar (Oxoid, UK) incubated at 37 ± 1°C. After incubation, 0.1 mL of each of the cultures were spread on the dried surface of Mueller-Hinton agar medium (Oxoid, UK). Resistance to antimicrobial agents was tested by commercially distributed disks (Oxoid, UK) with the following concentrations of antibiotics: 10 μg ampicillin, 10 UI penicillin, 1 μg oxacillin, 10 μg cefoxitin, 2 μg clindamycin, 15 μg erythromycin, 30 μg tetracycline, 30 μg novobiocin, 10 μg gentamicin and 30 μg vancomycin. The diameters of inhibition zones were measured after a 24-h-incubation at 37°C. Isolates were classified as susceptible, intermediately susceptible, or resistant [34].

The presence of erythromycin-induced clindamycin resistance was detected by the D-test as follows: a clindamycin disk (2 μg) was placed approximately 15 mm from the edge of an erythromycin disk (15 μg) on the Mueller-Hinton agar plate (Oxoid, UK) previously inoculated with the suspension of staphylococcal isolate tested. The test was considered positive if there was formation of a blunted or "D-shaped" zone around the disk containing clindamycin after a 18-h-incubation at 37°C [35].

2.6.1 Detection of β-lactamase activity

The presence of β-lactamase was tested with nitrocefin disks (Erba Lachema, Czech Republic), as the β-lactam ring of nitrocefin is susceptible to β-lactamase mediated hydrolysis. A colony on the Columbia blood agar (Oxoid, UK) was wiped across the nitrocefin disk (moistened with one drop of deionised water and held in forceps), and then observed for the development of a red colour. Degradation of nitrocefin was revealed by a rapid change in colour from yellow to red.

2.6.2 Detection of *mec*A gene

The presence of *mec*A gene was screened by a PCR method to detect the specific fragment of 527 bp [36]. *S. aureus* CCM 4750 (Czech Collection of Microorganisms Brno, Czech Republic) harbouring *mec*A gene was used as a positive control. A 25 μl reaction mixture was created containing 200 ng genomic DNA, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.8 μM of each primer, and 1.25 U *Taq* DNA polymerase (Qiagen, Germany). Two primers (Generi Biotech, Czech Republic) were used for the detection of *mec*A gene: Mecup1 (5´– GGGATCATAGCGTCATTATTC) and Mecup2 (5´– AACGATTGTGACACGATAGCC). Amplification conditions were as follows: initial denaturation at 94°C for 15 min, and 30 cycles – each consisting of (1) denaturation at 94°C for 45 s, (2) annealing at 55°C for 30 s, and (3) polymerisation at 72°C for 60 s. Final extension at 72°C for 10 min followed the last cycle. PCR products were analysed in a 2% agarose gel (Bioline, UK), stained with ethidium bromide (10 mg L^{-1}) and viewed by UV transillumination. Fragment size was determined by comparing with standard Marker C (SBS Genetech Co., Ltd., China). The obtained amplicons were further confirmed by sequencing using an Applied Biosystems 3130 Genetic Analyzer (USA).

2.6.3 Detection of PBP2a

An Oxoid PBP2´ Latex Agglutination Test Kit was used for detection of the altered penicillin binding protein (PBP2a) encoding resistance to oxacillin and all of β-lactam antibiotics in staphylococci. A reference strain of *Staphylococcus aureus* CCM 4223 (Czech Collection of Microorganisms, Brno, Czech Republic) was used as a positive control in this test. PBP2a was extracted according

to the manufacturer's instructions (Oxoid, UK). Presence of PBP2a was identified by visible agglutination in a drop of sensitized latex suspension within 3 minutes.

3 Results

Fifty-seven CoNS were isolated from 756 individual samples of public drinking water from the Slovak Republic (Table 2). The counts of staphylococci in the drinking water were generally very low – in individual samples they ranged from 3 to 8 CFU per 100 mL. Many of the staphylococci colonies had the same morphology, identical MALDI-TOF score values or score values below 2.000 (probable or not reliable genus identification), and were not tested further for the presence of virulence factors, production of enterotoxins or antimicrobial resistance. On average one species of staphylococci was typically isolated and tested further from each sample of water tested.

Drinking water was disinfected by chlorination. The amount of free chlorine in CoNS-positive samples ranged between 0.02 and 0.48 mg L^{-1}. Twenty-one samples of drinking water were not in compliance with the minimum free chlorine content (0.05 mg L^{-1}) required for water supply through the public distribution network in the Slovak Republic [37]. As seen in Table 2, both *S. caprae* and *S. chromogenes* were only isolated from less chlorinated samples of drinking water. Higher counts and more species of staphylococci were found in samples of drinking water with free chlorine contents between 0.05 and 0.30 mg L^{-1}. Species preferring normal levels of chlorination included *S. capitis* ssp. *urealyticus*, *S. cohnii* ssp. *cohnii, S. hominis* ssp. *hominis*, *S. xylosus*, *S. saprophyticus* ssp. *saprophyticus* and *S. epidermidis*. Free chlorine contents above the maximum limit (0.3 mg L^{-1}) significantly reduced the population of staphylococci. However, *S. haemolyticus* was also found in the only sample of drinking water where the limit of free chlorine content was significantly exceeded (0.48 mg L^{-1}).

The presence of some virulence factors typically found in CoPS were also found in numerous CoNS isolates (Table 3). Among the 57 isolates tested, 38.6% produced lecithinase, and 49.1% produced gelatinase. However, coagulase, hyaluronidase and DNase activities were not confirmed in any of the isolates tested. Six isolates (one strain of *S. warneri*, one strain of *S. chromogenes* and 4 strains of *S. haemolyticus*) did not show the presence of any virulence factor tested. Genes encoding the production of SED were only revealed in two CoNS isolates (*S. warneri* and *S. saprophyticus* ssp. *saprophyticus*).

Antimicrobial resistance testing of CoNS isolates from drinking water revealed that most isolates were resistant to ampicillin (66.7%), penicillin (64.9%), and erythromycin (57.9%), with less frequent resistance to oxacillin (40.4%), tetracycline and novobiocin (both 19.3%) (Table 4). Resistance to penicillin and ampicillin was confirmed in all isolates of *S. epidermidis* and *S. saprophyticus* ssp. *saprophyticus* and was predominant among *S. warneri* isolates (59.1%). However, the most frequent resistance found in isolates of *S. warneri* and *S. haemolyticus* was to erythromycin (63.6% and 69.2%, respectively). In contrast, isolates of *S. cohnii* ssp. *cohnii* and *S. hominis* ssp. *hominis* were sensitive to all antibiotics tested except for erythromycin. As shown in Table 4, none of the CoNS isolates were resistant to gentamicin, vancomycin, or clindamycin, although one isolate of *S. haemolyticus* possessing the *mec*A gene had intermediate resistance to gentamicin. Furthermore, a single isolate of *S. haemolyticus* (1.8%) had a positive

Table 2 Species of coagulase-negative staphylococci (CoNS) found in chlorinated drinking water with different free chlorine contents

Species of staphylococci	Number of isolates	Number of isolates from water with free chlorine contents of		
		0.02 – 0.04 mg L^{-1}	0.05 – 0.30 mg L^{-1}	0.48 mg L^{-1}
S. warneri	22	11	11	
S. haemolyticus	13	5	7	1
S. saprophyticus. ssp. *saprophyticus*	8	1	7	
S. capitis ssp. *urealyticus*	4		4	
S. epidermidis	4	1	3	
S. caprae	2	2		
S. cohnii ssp. *cohnii*	1		1	
S. chromogenes	1	1		
S. hominis ssp. *hominis*	1		1	
S. xylosus	1		1	
Total CoNS	57	21	35	1
Percentage (%)	100.0	36.8	61.4	1.8

Table 3 Virulence factors of coagulase-negative staphylococci (CoNS) isolated from samples of Slovakian drinking water

Species of staphylococci	Number of isolates	Number of isolates with production/presence of					
		Coagulase	DNase	Lecithinase	Gelatinase	Hyaluronidase	Gene *sed*
S. warneri	22	0	0	10	14	0	1
S. haemolyticus	13	0	0	3	4	0	0
S. saprophyticus. ssp. *saprophyticus*	8	0	0	1	5	0	1
S. capitis ssp. *urealyticus*	4	0	0	2	3	0	0
S. epidermidis	4	0	0	2	0	0	0
S. caprae	2	0	0	2	2	0	0
S. cohnii ssp. *cohnii*	1	0	0	1	0	0	0
S. chromogenes	1	0	0	0	0	0	0
S. hominis ssp. *hominis*	1	0	0	1	0	0	0
S. xylosus	1	0	0	0	0	0	0
Total CoNS	57	0	0	22	28	0	2
Percentage (%)	100.0	0.0	0.0	38.6	49.1	0.0	3.5

Table 4 Antimicrobial resistance of coagulase-negative staphylococci (CoNS) isolated from samples of Slovakian drinking water

Species of staphylococci	Number of isolates	Number of isolates resistant to										Number of isolates with			
		Ampicillin	Penicillin	Oxacillin	Cefoxitin	Gentamcin	Erythromycin	Clindamycin	Novobiocin	Tetracycline	Vancomycin	Positive D-test	β-lactamase	*mec*A gene	PBP2a
S. warneri	22	13	13	6	1	0	14	0	3	5	0	0	13	1	1
S. haemolyticus	13	6	6	3	3	0	9	0	0	4	0	1	6	3	2
S. saprophyticus. ssp. *saprophyticus*	8	8	8	7	1	0	3	0	7	1	0	0	8	0	0
S. capitis ssp. *urealyticus*	4	3	3	1	0	0	2	0	0	1	0	0	3	1	1
S. epidermidis	4	4	4	3	2	0	1	0	0	0	0	0	4	2	2
S. caprae	2	2	1	2	0	0	1	0	0	0	0	0	1	1	1
S. cohnii ssp. *cohnii*	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
S. chromogenes	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0
S. hominis ssp. *hominis*	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
S. xylosus	1	1	1	1	0	0	1	0	0	0	0	0	1	0	0
Total CoNS	57	38	37	23	7	0	33	0	11	11	0	1	37	8	7
Percentage (%)	100.0	66.7	64.9	40.4	12.3	0.0	57.9	0.0	19.3	19.3	0.0	1.8	64.9	14.0	12.3

result of the D-test, revealing erythromycin-induced resistance to clindamycin. Production of β-lactamase was observed in 64.9% of CoNS isolates. Results of latex agglutination screened the presence of the altered PBP2a in 7 isolates (30.4%), six of which showed simultaneous resistance to cefoxitin. However, *mec*A gene was detected in eight β-lactamase positive isolates of CoNS (34.8%) – one strain of *S. haemolyticus* was oxacillin- and cefoxitin-sensitive, one strain of *S. warneri* was oxacillin-sensitive but cefoxitin-resistant and another two isolates with *mec*A gene detected (*S. caprae* and *S. capitis* ssp. *urealyticus*) were resistant to oxacillin but were sensitive to cefoxitin. Two isolates of *S. epidermidis* were resistant to both oxacillin and cefoxitin, produced β-lactamase, had *mec*A gene and PBP2a. Therefore, they were identified as methicillin-resistant strains of *S. epidermidis* (MRSE).

Only a small subset of the CoNS isolates tested were sensitive to all anitibiotics, with the majority resistant to more than one. Eight isolates of CoNS (14.0%) did not show resistance to any of the ten antibiotics tested. These sensitive isolates included four strains of *S. haemolyticus* (30.8%), three strains of *S. warneri* (13.6 %) and one strain of *S. capitis* ssp. *urealyticus* (25.0%). Conversely, fourty-two of the CoNS isolates extracted from drinking water (73.7%) were simultaneously resistant to more than one antibiotic tested (Table 5), including isolates of *S. saprophyticus* ssp. *saprophyticus*, *S. capitis* ssp. *urealyticus*, *S. epidermidis*, *S. caprae*, *S. chromogenes*, *S. xylosus* as well as isolates of *S. haemolyticus* (69.2%) and *S. warneri* (63.6%).

Table 5 Numbers of staphylococcal isolates resistant to one and more antibiotics (Atbs) tested

Species of staphylococci	Resistance to					
	1 Atb	2 Atbs	3 Atbs	4 Atbs	5 Atbs	6 Atbs
S. warneri	5	2	5	4	2	1
S. haemolyticus	-	3	3	-	1	2
S. saprophyticus ssp. *saprophyticus*	-	-	1	4	2	1
S. capitis ssp. *urealyticus*	-	-	2	1	-	-
S. epidermidis	-	-	2	2	-	-
S. caprae	-	1	-	1	-	-
S. cohnii ssp. *cohnii*	1	-	-	-	-	-
S. chromogenes	-	-	1	-	-	-
S. hominis ssp. *hominis*	1	-	-	-	-	-
S. xylosus	-	-	-	1	-	-
Total CoNS (n = 57)	7	6	14	13	5	4
Percentage (%)	12.3	10.5	24.6	22.8	8.8	7.0

Multiresistance (i.e. resistance to three or more classes of antimicrobial agents [38]) was confirmed in 8 out of 57 CoNS isolates (14.0%). Two isolates (*S. warneri* and *S. capitis* ssp. *urealyticus*) were resistant to ampicillin, penicillin, erythromycin and tetracycline. One isolate of *S. warneri* was resistant to ampicillin, penicillin, oxacillin, novobiocin and tetracycline and another isolate of *S. saprophyticus* ssp. *saprophyticus* was resistant to ampicillin, penicillin, oxacillin, erythromycin and novobiocin. Two other isolates (*S. warneri* and *S. saprophyticus* ssp. *saprophyticus*) were resistant to ampicillin, penicillin, oxacillin, erythromycin, novobiocin and tetracycline. Finally, two isolates of *S. haemolyticus* were both resistant to ampicillin, penicillin, oxacillin, cefoxitin, erythromycin and tetracycline.

4 Discussion

This study is one of only a small number of past studies to isolate CoNS species from drinking water. Although staphylococci are ranked among four bacterial genera with high dominance in samples of potable water [39], CoNS are not usually identified to the species level in routine laboratories [7]. This is perhaps why so little has been published about CoNS species in drinking water. The occurrence of staphylococci has mainly been reported in polluted surface water [40] or in the biofilm from hospital wastewater [41]. In this study, eight out of ten species of coagulase-negative staphylococci identified (except for *S. caprae* and *S. chromogenes*) were also isolated from samples of drinking water and wastewater in Portugal [42]. The authors of the Portuguese study report dominance of *S. pasteuri* and *S. epidermidis* in samples taken from a drinking water treatment plant as well as from a drinking water distribution network. They also found prevalence of *S. saprophyticus* in samples taken from a wastewater treatment plant. Investigation of biofilms occurring in a public drinking water distribution system in Romania revealed the presence of *S. warneri* and *S. vitulinus* [43]. Based on results of those studies, *S. epidermidis* – an important nosocomial opportunistic pathogen [44,45] – is the most frequently isolated species among staphylococci occurring in the aquatic environment.

Although virulence factors are mainly associated with coagulase-positive *S. aureus*, they were also associated with many of the coagulase-negative species found in Slovakian drinking water. This is not the first study to document presence of virulence factors in CoNS [46-49]. To distinguish between pathogenic CoPS and non-pathogenic coagulase-negative species, *clumping factor*, DNase and hyaluronidase are of great importance. However, some of those factors were also found in clinical and food isolates of CoNS [48, 50-53]. Results of our study demonstrate the absence of coagulase, DNase and hyaluronidase production, with lecithinase activity confirmed in 38.6% and gelatinase activity confirmed in 49.1% of CoNS isolates. Gelatinase is a proteolytic enzyme that allows microorganisms to hydrolyse gelatin, fibrinogen and collagen. Staphylococci often participate in wound infections and this enzymatic activity helps them to penetrate deeper into connective tissues. As the importance of CoNS has been recognized in human infections earlier than in animal infections and food poisoning, most CoNS research has been performed in human cases [54]. Gelatines production was reported in 67.27% of clinically important isolates of staphylococci from patients with coxarthrosis and gonarthrosis [47].

The presence of one or more virulence factors was reported in 77.8% of the CoNS strains isolated from the skin of hospitalized newborns [48], and were found to be involved in the production of slime (17.1%), haemolysins (19.6%), lipase (17.1%), lecithinase (3.4%), DNAse (15.4%), thermonuclease (7.7%), and enterotoxins A, B or C (37.6%). The occurrence of virulence factors in clinical CoNS isolates is more frequent and more diversified as compared with strains we isolated from chlorinated drinking water, possibly due to the less hospitable environment of the drinking water (e.g. lower temperature, and fewer nutrients leading to less bacterial competition).

As found in past studies [47], CoNS species isolated in this study were resistant to several antibiotics. Antimicrobial resistance is more common in clinically important isolates of CoNS, with most frequent resistance to penicilin (84%), erythromycin (58%), and oxacillin (55%), followed by gentamicin (20%) and tetracycline (16%). In contrast, none of the 57 CoNS isolates tested in the present study were resistant to gentamicin. Another study which isolated CoNS from Portuguese water supplies [42] found that antibiotic-resistant CoNS may colonize different types of water, including drinking water that fulfills all the quality standards. In agreement with our results, the Portuguese study also found CoNS species were resistant to β-lactams, tetracycline, clindamycin and erythromycin, the last of which was highly prevalent (34.7%). In most erythromycin-resistant CoNS isolates they confirmed the presence of the *msr*A gene, which encodes an efflux pump for this antibiotic. The Portuguese study did not reveal resistance to vancomycin, gentamicin, sulfamethoxazole-trimethoprim or ciprofloxacin in any of 172 isolates of staphylococci from a drinking water distribution network, responsible for supplying water to the consumers. Whereas 25% of the CoNS isolates from Portuguese drinking water had simultaneous resistance to two or more antibiotics, the present study found multiresistance in 14% of the isolates (i.e. three strains of *S. warneri*, two strains of both *S. haemolyticus* and *S. saprophyticus* ssp. *saprophyticus*, and a single strain of *S. capitis* ssp. *urealyticus*).

This is the first study to test methicillin/oxacillin resistance among coagulase-negative staphylococci isolated from drinking water. The presence of the *mec*A gene was only reported in *S. epidermidis* present in biofilms from hospital wastewater [41]. However, screening for *mec*A gene in our study revealed its presence in five out of ten species of CoNS (*S. haemolyticus*, *S. epidermidis*, *S. warneri*, *S. caprae*, and *S. capitis* ssp. *urealyticus*). Among eight isolates of CoNS possessing the *mec*A gene, seven also had altered PBP2a and four were simultaneously resistant to oxacillin and cefoxitin. Sensitivity to oxacillin or cefoxitin in the *mec*A gene-positive isolates could probably be explained by the presence of "dormant genes" which remain inactive *in vitro*, and genetically encoded proteins are not synthesized because of the block in their transcription; or, in the case of PBP2a, the altered protein could be insufficiently manifested and, therefore, not detected by latex agglutination. Environmental bacteria are not constantly in contact with antibiotics; therefore, repressing their resistance genes is probably advantageous [55].

The presence of methicillin-resistant CoNS in Slovakian drinking water is of particular concern as they could spread multidrug resistance to the environment [25,26]. In the present study, production of β-lactamase was confirmed in each out of 8 methicillin-resistant, as well as in 30 methicilline-sensitive CoNS isolates. This is significant because β-lactamases produced by some bacteria provide resistance to β-lactam antibiotics. Based on results of both phenotypic and genotypic analyses, two isolates of *S. epidermidis* (50%) were identified as methicillin-resistant. It is generally accepted that such strains of MRSE increasingly participate in the spread of multidrug resistance to the environment [25,26]. Except for drinking water, *mec*A gene has only been previously detected in 4 out of 50 (8.0%) CoNS (one strain of *S. simulans* and three strains of *S. haemoylticus*) isolated from water in swimming pools [56].

The detection of enterotoxin encoding genes in CoNS species isolated from drinking water is also concerning, given the potential health hazards of enterotoxins, but these results need to be explored further. Currently, little is known about the enterotoxigenic potential of coagulase-negative staphylococci, but the enterotoxigenic properties of CoNS cannot be ignored [54] and CoNS should be considered harmful for human health based on their enterotoxigenic potential [57]. Several reports related to food and dairy products confirmed that CoNS can produce enterotoxins [58,59]. In a study of healthy goats [58], 22% of the CoNS species (including *S. chromogenes*, *S. warneri, S. sciuri*, *S. saprophyticus*, and *S. lentus*) were able to secrete enterotoxins. Furthermore, coagulase-negative staphylococci are thought to be an important reservoir of virulence-associated genes that significantly contribute to the evolution of *S. aureus* in both community and hospital settings [60]. Although they are proteins, staphylococcal enterotoxins are resistant to heat and most proteolytic enzymes [61]. Staphylococcal enterotoxins are also superantigens which are encoded by accessory genetic elements and can be transferred horizontaly not only among staphylococcal strains but also between

bacterial species [62]. In the current study, genes encoding production of SED were only confirmed in two isolates (3.5 %) of CoNS from drinking water. However, detection of SE genes without determining the status of their expression should be taken with caution [54].

Chlorination is the most applied disinfection process in water treatment but it only seems to be an affective treatment against CoNS in Slovakian drinking water samples in higher concentrations. Chlorine oxidizes the germ cells, alters cell permeability, inhibits enzymatic activity and damages the cell DNA and RNA [63]. However, the effect of chlorine on bacterial DNA may only be achieved for quite high disinfectant doses typically used in wastewater disinfection [64]. The process of chlorination has mainly been studied in Gram-negative bacteria [65-67], with chlorine doses of 0.5-5.0 mg L^{-1} having no effect on plasmid DNA of antibiotic-resistant bacteria [68]. Unfortunately, bacteria injured by disinfection processes can survive and re-grow at low chlorine doses [69-70]. Results of the present study show that CoNS may colonize drinking water processed with different chlorine concentrations. However, a higher level of chlorination significantly reduced both the number of positive samples as well as the spectrum of staphylococcal species. Currently, there are no data available on the effects of water chlorination on survival of CoNS but since metagenomic analysis has revealed the prevalence of antibiotic resistance genes in drinking water it suggests that chlorination helps concentrate antibiotic resistance genes in drinking water, which might represent health risks for the consumers [67].

The results of this study suggest that CoNS in drinking water could be dangerous to humans and animals if they were at high enough concentrations, but further research is needed to establish the true risk they repesent. Staphylococci are ranked among autochthonous, heterotrophic plate count (HPC) bacteria which are commonly found in drinking water but there is currently a lack of clinical and epidemiological evidence that elevated populations or specific genera within this group of bacteria pose an increased health risk to human populations. Therefore, no health-based standards for HPC bacteria in drinking water have yet been established [71]. Results of this study showed that the counts of CoNS in drinking water are generally very low. However, in addition to its consumption, drinking water is widely used for many other purposes (washing, bathing, or cleaning). Therefore, CoNS can easily come into contact with the skin and mucous membranes of both humans and animals. When harbouring genes encoding virulence factors or resistance to antibiotics, CoNS may serve as donors, increasing pathogenic potential of the normal skin microflora including pathogenic coagulase-positive staphylococci. Furthermore, the occurrence of antibiotic resistant CoNS in water destined for human consumption may represent a hazard under conditions capable of favouring their overgrowth (e.g. biofilm formation or antibiotherapy) [42]. Therefore, more intensive research is required in this field to reveal a real pathogenic potential of CoNS in chlorinated drinking water.

5 Conclusion

This study revealed the presence of antibiotic resistant coagulase-negative staphylococci in chlorinated drinking water distributed through the public water supply network in the Slovak Republic. Despite their low incidence (7.5 %), the presence of some virulence factors (production of gelatinase, lecithinase, and β-lactamase), *mec*A gene, antimicrobial resistance, as well as gene-encoded ability to produce enterotoxins in the CoNS species found could make them a health concern. Although the spectrum of CoNS species found in drinking water was broader than reported for food and clinical CoNS isolates, phenotypic expression of virulence factors was less frequent and less extensive, potentially because chlorinated drinking water is a less suitable environment (i.e. lower temperatures and fewer nutrients do not require strong and comprehensive enzymatic activity, and thus there may be no selective pressure), resulting in significantly reduced bacterial competition. As demonstrated in this study, antibiotic resistant CoNS species are entering the water supply system, which is subsequently distributed to a wide range of consumers including immuno-compromised persons, newborns, or patients in hospitals. Recovery of two methicillin-resistant *S. epidermidis* strains (MRSE) from drinking water should be of particular concern. Further reasearch is needed to fully assess the risks these CoNS species represent but it is clear from these results that more attention should be paid to their pathogenic potential in drinking water to eliminate possible health risks for consumers in the future.

Conflict of interest: Authors delcare nothing to disclose.

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