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SHORT COMMUNICATION

Disc-diffusion and PCR Detection of Methicillin Resistance in Environmental Airborne Strains of *Staphylococcus* spp.

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Abstract

The aim of this study was to assess the species composition of airborne *Staphylococcus* spp. in public premises, to determine the methicillin resistance of the isolates and the prevalence of *mecA* gene, determining resistance to β-lactams. In total 65 *Staphylococcus* strains were isolated from 54 sites. Four strains exhibited phenotypic methicillin resistance, while the presence of *mecA* gene was found in 11 strains. The results of both assays were compared, showing that the phenotypic tests revealed methicillin resistance only in 36% of the examined samples. This study revealed high species diversity among airborne *Staphylococcus* spp. population, which consists of multidrug resistant strains.

Key words: airborne *Staphylococcus* spp., disc-diffusion method, *mec*A gene, methicillin resistance, PCR

Staphylococci are among the most frequently isolated human pathogenic bacteria, causing nosocomial infections, acute infections and in extreme cases, death of patients. Over many years, the attention of microbiologists and clinicians over the world was focused on *Staphylococcus aureus*, as a major factor of nosocomial infections. Currently, increasing attention is paid to coagulase-negative staphylococci (CoNS) that were considered harmless until 1970 (Kloos and Banerman, 1994; von Eiff *et al.*, 2001). Data from the United States National Nosocomial Infections Surveillance System from January 1990 to May 1999 showed that CoNS were the most commonly reported pathogens (37.3% for CoNS versus 12.6% for *S. aureus*) isolated from bloodstream infections in intensive care unit patients (American Journal of Infection Control, 1999). CoNS are also among the most frequently isolated bacteria in clinical microbiology laboratories (Patrick, 1990). More importantly, CoNS often serve as reservoirs of antimicrobial resistance determinants, since they usually have a high prevalence of multidrug resistance (Bastos *et al.*, 1999). Therefore, it is important to characterize CoNS strains isolated from the environment. Methicillin resistance of *Staphylococcus* spp. is determined by the presence of the *mec*A gene, encoding the protein PBP2a (*penicillin binding protein*) (Chambers, 1997). PBP2a protein, unlike the PBP protein is not inactivated by the β-lactam antibiotics. The PBP2a protein, despite the presence of antibiotics, can continuously participate in the synthesis of the bacterial cell wall, thus contributing to resistance to *e.g.*: penicillins, cephalosporins, monobactams, carbapenems and other drugs associated with β-lactamase inhibitors (Georgopapadakou, 1993; Idzik *et al.*, 2000). Drug resistance, including resistance to methicillin is routinely evaluated in diagnostic laboratories using a disc-diffusion method (EUCAST, 2012). However, as shown by Idzik *et al.* (2000), strains phenotypically identified as sensitive, can possess the *mec*A gene, responsible for resistance to β-lactam antibiotics. Similarly, in staphylococci exhibiting resistance in a disc-diffusion test, the presence of *mec*A gene may not be detected, which in turn may indicate the existence of other, more complex mechanisms of resistance to β-lactam antibiotics (Banerjee *et al.*, 2010). From an epidemiological point of view, this phenomenon is very dangerous and requires monitoring.

Therefore, the aim of this study was to isolate and identify the bioaerosol-forming species of *Staphylococcus* spp. in public premises. Another aim was to assess the drug resistance of the isolated strains, with particular emphasis on methicillin resistance and to compare the results of phenotypic tests with molecular detection of *mec*A gene, responsible for the resistance to β-lactam antibiotics. The air sampling was conducted in 45 public utility buildings in the city of Kraków, divided into 4 groups: teaching facilities of the University of

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Table I
Location and characteristics of the study sites

Location					
TEACHING FACILITIES	CHURCHES	SHOPPING MALLS	HEALTH CARE FACILITIES	CZERNICHÓW	CONTROL
1U Laboratory classroom	1K Capuchin monastery	1G Krakowska	1S Jagiellońskie Est. Clinic	6CZ Farm premise – horses	1 Jordan's Park
2U Elevator	2K Śt. Anna church	2G Kazimierz	2S Złotego Wieku Est. Clinic	7CZ Farm premise – cows	2 Planty meadow
3U Toilet	3K Franciscan monastery	3G Plaza	3S Rydygiera Hospital	9CZ Health care clinic	3 Polish Aviators Park
4U Vivarium	4K Dominican monastery	4G M1	4S Urocze Est.	10CZ Farm premise Clinic	4 Botanical Garden – calf house
5U Reading room	5K Śt. Barbara church	5G Carrefour	5S Szkolne Est. Clinic		4CZ Czernichów Park
6U Bar	6K Lord's Ark church	6G Leroy Merlin	6S Żeromskiego Hospital		
7U Gym	7K Our Lady of Częstochowa churchi	7G Tesco	7S Borek Fałęck Est. Clinic		
8U Lecture hall 1	8K Łagiewniki Sanctuary	8G Biedronka	8S Neurology CMUJ		
9U Lecture hall 2	9K Immaculate Conception of Virgin Mary church	9G Solvay	9S Gynecology CMUJ		
10 U Corridor	10K Sacred Heart of Jesus church	10G Zakopianka	10S Occupational Medicine		
11U Locker room	11K St. Mary's Basilica	11G Bonarka			
12U Deanery					
13U PCR lab					

Agriculture and the Jagiellonian University, churches, shopping malls and health care facilities and in 4 sites located outside the city in Czernichów. Five sites located outdoors were used as control – 4 in Kraków and 1 in Czernichów (Table I).

Air sampling was performed twice – in December 2012 and June 2013 using a MAS-100 impactor (Merck) in order to verify the seasonal effect on the species composition of *Staphylococcus* spp. Two types of media were used for isolation of *Staphylococcus* bacteria: general medium – Tryptic Soy Agar – TSA (bioMerieux) supplemented with 5% sheep blood and differential Baird-Parker medium (bioMerieux) for microorganisms belonging to the genus *Staphylococcus* and *Micrococcus*. Species identification of *Staphylococcus* spp. was performed according to Kloos and Schleifer (1975), Kloos and Bannerman (1994), Gaillot *et al.* (2000) and Murray (2007). Gram stained microscopic preparations were made from macroscopically characteristic colonies that grew on TSA and Baird-Parker media. Another stage was the furazolidone sensitivity test to discriminate between the genus *Staphylococcus* and *Micrococcus*. Bacteria susceptible to furazolidone were identified as *Staphylococcus,* while the remaining ones – as *Micrococcus*. Subsequently, the catalase test was performed in order to exclude the presence of catalase-negative streptococci in the examined material. By using free coagulase, the *Staphylococcus* strains were differentiated into coagulase-positive and coagulase-negative. The final step was the biochemical test performed using the API Staph system (bioMerieux). This was followed by the assessment of antibiotic sensitivity of all examined strains using a disc-diffusion method on Mueller-Hinton Agar II (bioMerieux) according to the recommendations of EUCAST (2012). The following antibiotics were used in the study: cefoxitin, erythromycin, clindamycin, cotrimoxazole, doxycycline, ciprofloxacin, gentamicin. Reference strains *S. aureus* ATCC 25923 (methicillin-sensitive) and *S. aureus* MR 3 (methicillin-resistant, strain derived from the culture collection of the Jan Bober Centre for Microbiological research and Autovaccines) were used as control. Prior to DNA extraction, the bacterial strains were cultured on TSA supplemented with 5% sheep blood at 37°C for 24 hours. After the incubation, 4–5 colonies were collected with a sterile loop and suspended in 100 µl Tris buffer. Bacterial DNA was extracted using Genomic Mini DNA extraction kit (A&A Biotechnology, Poland), following the manufacturer's instructions. PCR reactions were performed using primers *mec*A-F (5'GTAGAAATGACTGAACGTCCGATAA3') and *mec*A-R (5'CCAATTCCACATTGTTTCGGTCTAA3')

and the expected product length was 310 bp (Geha *et al.,* 1994). Polymerase chain reaction contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1 × PCR buffer and 1 U DreamTaq DNA polymerase in a total volume of 25 µl. The following temperature profile was used for DNA amplification: initial denaturation at 94°C for 2 min followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) and final extension at 72°C for 5 min. PCR amplifications were performed using T100™ Thermal Cycler (Bio-Rad, USA). The PCR products were visualized by 1 × TBE electrophoresis in ethidium-bromide-stained, 1% agarose gel. The PCR reaction was performed on all examined environmental strains together with the reference strains.

In total, 65 *Staphylococcus* strains were isolated from 54 sampling sites. 15 species of coagulase-negative staphylococci were identified, while *S. aureus* was not detected in the examined material. Two species, *i.e. Staphylococcus haemolyticus* and *Staphylococcus* hominis dominated among the collected strains (Table II).

Table II
Prevalence of the collected *Staphylococcus* species

No.	Species	Number of isolates	No.	Species	Number of isolates
1.	*S. haemolyticus*	12	9.	*S. lentus*	2
2.	*S. hominis*	10	10.	*S. saprophyticus*	2
3.	*S. epidermidis*	8	11.	*S. simulans*	2
4.	*S. warneri*	6	12.	*S. kloosii*	2
5.	*S. xylosus*	5	13.	*S. equorum*	2
6.	*S. cohnii cohnii*	5	14.	*S. auricularis*	1
7.	*S. capitis*	4	15.	*S. caprae*	1
8.	*S. cohnii ureal*	3			

Among 65 isolates, 42 (65%) exhibited resistance to at least one of the analyzed antibiotics in the disc-diffusion test. Four strains (6%) were resistant to cefoxitin, which indicates the methicillin resistance of those isolates (Żabicka and Hryniewicz, 2010). Two of those strains were isolated in the summer and two in the winter; they belonged to different species and showed varied resistance to other tested antibiotics. The only common feature in the methicillin-resistant strains was their origin, *i.e.* 3 of 4 strains were isolated from the air of Kraków shopping malls and one from the hospital (Table III). During the winter isolation, despite unfavorable meteorological conditions (low air temperature, low humidity), 39 strains were collected, while 26 strains were isolated in the summer.

The PCR test revealed that 11 (17%) out of 65 strains possessed *mec*A gene which determines resistance to β-lactam antibiotics. The results of phenotypic analysis were compared with those obtained in PCR reaction (Table III). Based on the conducted study, it can be concluded that possessing the gene that determines the methicillin resistance is not synonymous with its detection by the disc-diffusion method. The results of both methods were congruent only in the case of 4 (36%) out of 11 *Staphylococcus* spp. strains. It should be noted that the methicillin-resistant strains originated from densely populated premises (shopping mall and churches). On the other hand, methicillin-resistant staphylococci were not detected in control sites (parks and gardens). Microbiological quality of indoor air is the subject of many studies worldwide (Wanner *et al.,* 1993; Lee and Jo, 2006). This is due to the fact that with the development of civilization, people spend more time indoors (87% of time). Therefore, it has become important to understand the potential threat posed by the bioaerosol inside public facilities (Gąska-Jedruch and Dudzinska, 2009). Staphylococci are commonly isolated from the air in residential and commercial buildings (Gąska-Jedruch and Dudzinska, 2009) but also from the outdoor air (Zmysłowska and Jackowska, 2005). The most common indoor species are: *Staphylococcus epidermidis*, *S. haemolyticus*, *Staphylococcus saprophyticus*, *Staphylococcus auricularius*, *Staphylococcus. capitis*, *Staphylococcus warneri*, *S. hominis* (Bonetta *et al.*, 2010). On the other hand, outdoor air is a reservoir of among others: *Staphylococcus xylosus, Staphylococcus lentus, S. aureus* and *S. epidermidis* (Zmysłowska and Jackowska, 2005). People staying indoors are exposed to continuous contact with bacteria of the genus *Staphylococcus*, which is a disturbing phenomenon, as infections caused by both coagulase-positive and coagulase-negative methicillin-resistant staphylococci become increasingly frequent (Idzik *et al.,* 2000). Sensitivity and speed of previously used methods for the determination of drug resistance is insufficient, therefore fast, highly specific and sensitive techniques, such as the PCR, are being sought (York *et al.,* 1996). The PCR technique, which was used in this study to determine the presence of *mecA* gene, increased the detection rate of the methicillin-resistant isolates. Only in 36% of cases the methicillin resistance was detected using both methods employed in this study. Our results are congruent with those obtained by other authors, who also observed very low correlation between the results of phenotypic tests and molecular analyses (York *et al.*, 1996; Idzik *et al.*, 2000). This is very alarming, because this situation creates the possibility of missing strains, that can be a source of fateful infections. As shown in this research, strains forming a microbial aerosol at public premises, belonging to the species until recently considered as little virulent, are carriers of methicillin resistance genes. Additionally, based on the conducted study it can be assumed

Table III
Drug resistance of the collected *Staphylococcus* spp. strains

No.	Collection date (winter/summer)	Sampling site	Antibiotic resistance	mecA gene (+/–)	Species
1.	W	1U	Erythromycin	–	*S. warnerii*
2.	W	2U	Erythromycin	–	*S. haemolyticus*
3.	W	4U	–	+	*S. lentus*
4.	W	4U	–	–	*S. xylosus*
5.	S	4U	–	–	*S. lentus*
6.	W	7U	–	+	*S. hominis*
7.	W	7U	Erythromycin Clindamycin	–	*S. saprophyticus*
8.	W	9U	Erythromycin	–	*S. cohni cohni*
9.	W	10U	Erythromycin Doxycycline	–	*S. epidermidis*
10.	W	10U	–	–	*S. xylosus*
11.	W	12U	–	–	*S. warnerii*
12.	W	13U	–	+	*S. cohni cohni*
13.	W	1K	Erythromycin Clindamycin	–	*S. xylosus*
14.	S	2K	Erythromycin	–	*S. kloosii*
15.	S	3K	–	–	*S. capitis*
16.	W	4K	–	–	*S. hominis*
17.	W	4K	–	–	*S. xylosus*
18.	W	5K	Erythromycin	–	*S. epidermidis*
19.	W	5K	–	–	*S. warnerii*
20.	S	5K	–	–	*S. caprae*
21.	S	8K	Cotrimoxazole	+	*S. epidermidis*
22.	W	8K	Erythromycin	–	*S. haemolyticus*
23.	W	8K	Erythromycin	–	*S. hominis*
24.	W	10K	–	–	*S. epidermidis*
25.	W	10K	Erythromycin	–	*S. hominis*
26.	S	4CZ	–	–	*S. equorum*
27.	S	6CZ	Erythromycin Doxycycline Ciprofloxacin	–	*S. cohni ureal*
28.	S	7CZ	Erythromycin	–	*S. cohni ureal*
29.	S	7CZ	Erythromycin	–	*S. cohni ureal*
30.	S	9CZ	–	–	*S. hominis*
31.	S	9CZ	Cotrimoxazole Gentamicin	–	*S. epidermidis*
32.	S	10CZ	–	–	*S. xylosus*
33.	S	1S	Erythromycin Cotrimoxazole	–	*S. saprophyticus*
34.	W	1S	Erythromycin	–	*S. cohni cohni*
35.	W	2S	Erythromycin	–	*S. warnerii*
36.	S	2S	–	+	*S. hominis*
37.	W	2S	Erythromycin	–	*S. haemolyticus*
38.	W	2S	Erythromycin Clindamycin Doxycycline	–	*S. simulans*
39.	S	4S	Erythromycin	–	*S. haemolyticus*
40.	W	4S	–	–	*S. kloosii*
41.	W	4S	–	–	*S. haemolyticus*
42.	W	5S	Erythromycin	–	*S. capitis*
43.	W	6S	Erythromycin	–	*S. warnerii*
44.	S	7S	Erythromycin	–	*S. hominis*
45.	W	8S	Erythromycin Clindamycin	–	*S. equorum*
46.	W	9S	Cefoxitin Erythromycin Cotrimoxazole Doxycycline	+	*S. cohni cohni*
47.	W	10S	Erythromycin	–	*S. epidermidis*

Table III continued

No.	Collection date (winter/summer)	Sampling site	Antibiotic resistance	mecA gene (+/–)	Species
48.	S	10S	–	+	*S. haemolyticus*
49.	W	1G	Clindamycin	–	*S. haemolyticus*
50.	W	2G	Cefoxitin Erythromycin	+	*S. hominis*
51.	W	2G	Erythromycin	–	*S. hominis*
52.	S	2G	Cefoxitin	+	*S. haemolyticus*
53.	S	3G	Erythromycin	–	*S. epidermidis*
54.	W	4G	Erythromycin	–	*S. haemolyticus*
55.	W	4G	Erythromycin	–	*S. haemolyticus*
56.	S	5G	–	–	*S. capitis*
57.	W	6G	Erythromycin	–	*S. warnerii*
58.	W	6G	–	–	*S. auricularis*
59.	S	7G	Erythromycin	–	*S. haemolyticus*
60.	S	8G	Erythromycin	–	*S. cohni cohni*
61.	S	9G	Cefoxitin Erythromycin Cotrimoxazole	+	*S. simulans*
62.	S	10G	Erythromycin	–	*S. epidermidis*
63.	W	10G	Erythromycin	–	*S. hominis*
64.	S	11G	Erythromycin Doxycycline	+	*S. haemolyticus*
65.	W	11G	–	–	*S. capitis*
66.	Positive control		Cefoxitin	+	*S. aureus* MR 3
67.	Negative control		–	–	*S. aureus* ATCC 25923

that the absence of methicillin-resistant staphylococci in outdoor air, in contrast to the indoor air, may be related to the high population density of the examined premises. It is therefore well known that people are the primary source of microbiological contamination indoors (Gołofit-Szymczak and Skowron, 2005).

In conclusion, the air in public premises is a reservoir of numerous, various *Staphylococcus* species, that are resistant to multiple antibiotics, including cefoxitin, which indicates their methicillin resistance. PCR detection revealed that the strains that did not exhibit methicillin resistance in phenotypic analysis, may possess the *mecA* gene, responsible for the formation of resistance mechanisms. Therefore, it is necessary to monitor the abundance of potentially pathogenic bacteria in the environment, as they may become an important but frequently ignored etiological infection agent in humans.

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