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Drug resistance profile and detection of genes responsible for methicillin resistance in *Staphylococcus aureus* isolated from municipal waste

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Abstract: Currently, we are facing the ever-increasing phenomenon of bacteria being resistant to antibiotics. It is the consequence of excessive and incorrect use of drugs. The phenomenon is a global problem affecting bacteria both in their hospital population and in the natural environment. Municipal waste is an environment conducive to the development of microorganisms, therefore it contains various groups of bacteria, including drug-resistant staphylococci. The aim of the study was to identify species of bacteria, determine their antibiotic resistance, and assess the occurrence of genes responsible for methicillin resistance in *Staphylococcus aureus* isolated from mixed municipal waste. Strains were isolated by Koch's serial dilution method with the use of microbiological media. Species were identified using the MALDI TOF-MS technique, whereas the drug resistance profile was determined by disk diffusion and molecular PCR methods. 250 isolates of *S. aureus* were collected. The highest resistance found was to cefoxitin, erythromycin and tetracycline. Among the bacteria collected, resistance to 1, 2, 3 or 4 antibiotics at the same time was the most common, with a maximum of 10. Additionally, 45 (18%) MDR (multidrug-resistant) isolates were detected. Methicillin resistance was found by the disk diffusion test in 60 (24%) strains, while the *mecA* gene was detected in as many as 180 (72%) isolates.

Keywords: drug resistance, *mecA* gene, methicillin resistance, municipal waste, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus spp. commensal bacteria is commonly isolated from skin, mucous membranes, and skin glands of mammals. Due to their ability to produce coagulase, these bacteria have been divided into coagulase-positive staphylococci (CPS), considered much more virulent, and coagulase-negative staphylococci (CNS) [Layer *et al.* 2007]. The leading role of staphylococci in pathogenesis was first recognised in medicine of human. Hence a lot of studies on the toxicity of these microorganisms focused on strains related to human diseases. On the other hand, they are also an etiological factor of food poisoning, cause eye and wound infections or infections of the reproductive and respiratory systems in animals [Podkowik *et al.* 2013]. The best known and characterised representative of coagulase-positive staphylococci is *Staphylococcus aureus* [Wolny-Koładka *et al.* 2014]. *S. aureus* is considered one of the most important etiological factors of nosocomial infections. Staphylococcal infections, due to their ease to spread and transmit from human to human and between humans and animals, pose a threat to public health [Bhargava, Zhang 2012].

The rise and spread of drug resistance among staphylococci is a serious global problem. In particular, the increasing bacterial resistance to routinely and commonly used antibiotics for staphylococcal infections is of legitimate concern [Wolny-Koładka *et al.* 2014]. Methicillin-resistant *S. aureus* (MRSA) invariably remain of close interest to scientists worldwide due to their risk to animals and humans. Resistance to methicillin (cefoxitin resistance) is described as resistance to β-lactam antibiotics, i.e. cephalosporins, penicillins, monobactams and carbapenems [Huber *et al.* 2011]. The presence of the *mecA* gene in the staphylococcal genome, located within the staphylococcal cassette chromosome *mec* (SCC*mec*), determines methicillin resistance. SCC*mec* are mobile genetic elements (MGE), which

can be transferred horizontally between strains of the same or different species [Hanssen, Ericson Solilid 2006]. The *mecA* gene encodes the PBP2a (penicillin binding protein) that, unlike the PBP, is not inactivated by β-lactam antibiotics. The PBP2a protein, even in the presence of an antibiotic, can continuously participate in the synthesis of the bacterial cell wall, effectively contributing to antibiotic resistance [Hiramatsu *et al.* 2002].

Municipal landfills are a potential place where drug-resistant *S. aureus* strains occur [Velsivasakthivel, Nandini 2014]. Municipal solid waste (MSW) from landfills includes household waste, as well as medical waste, agricultural waste, food waste and any other waste of anthropogenic origin [Anand *et al.* 2021]. Solid waste contributes to the growth of mixed microbial populations. Several factors are conducive to the process, including its heterogeneous nature and structure, surface on which microorganisms thrive, high content of organic and inorganic nutrients easily assimilated by microorganisms, as well as appropriate humidity and temperature. The latter are usually higher than on the surface of soil. Waste forms a very heterogeneous environment consisting of a wide range of organic particles of natural and xenobiotic origin, and some or all of its components can be used by microorganisms [Barlaz, Palmisano 1996]. Since undesirable components, such as antibiotics (including β-lactam antibiotics), bioactive waste and disinfectants also end up in the waste biomass due to inadequate segregation and treatment, there is a selective pressure in landfills, favouring the build-up of microbial drug resistance. Therefore, landfills become potential sites for the emergence and spread of antibiotic resistance genes (ARGs). Hazardous landfill leachate, containing both drug-resistant bacteria and antibiotic resistance genes, as well as the antibiotics themselves, can percolate into the environment and pose a threat to public health [Chen *et al.* 2017; You *et al.* 2018].

Multidrug-resistant staphylococci (MDR), resistant to at least 3 classes of antibiotics at the same time [Maddox *et al.* 2012], are responsible for infections in humans and animals and are a very disturbing threat from an epidemiological point of view [van Duijkeren *et al.* 2010]. Medicine uses many antibiotics of different classes. Hence, antibiotic-resistant staphylococci may also be selected among strains included in the physiological flora [Wolny-Koładka 2018]. Given the fact that waste is a source of many types of pathogenic microorganisms, including staphylococci [Pawłat *et al.* 2021; Wolny-Koładka *et al.* 2021], it should be assumed that it is also a reservoir of drug-resistant microorganisms and thus poses a threat to people dealing with waste storage and processing. Moreover, there is a danger of transmitting pathogenic strains outside the premises of industrial plants and enterprises that store municipal waste. This poses a serious threat to public health and also leads to the uncontrolled spread of pathogenic bacteria in the environment [Cyprowski *et al.* 2019]. For this reason, it is necessary to monitor the increasing bacterial resistance to antibiotics and to learn about its molecular basis. Such an approach is a chance to develop effective and rational schemes aimed at controlling or limiting the spread of drug resistance among diverse components of the ecosystem [Angulo *et al.* 2004].

Previous publications in this area have focused on methicillin-resistant *S. aureus* isolated from humans, animals and the hospital environment [Jaradat *et al.* 2020; Rahman *et al.* 2018]. To the best of our knowledge, there are no publications on the isolation and determination of such microorganisms from municipal waste. Taking into account the fact that the diversity of municipal waste has been growing every year, we can assume that the amount of drug-resistant *S. aureus* present has increased as well. Therefore, the research helps to shed new light on the presence of methicillin-resistant staphylococci in waste and the potential spread of drug resistance in the environment.

The aim of the study is to identify species of *Staphylococcus aureus* isolated from municipal waste. The susceptibility of the strains to antibiotics is assessed, with particular emphasis on phenotypic and genetic detection of resistance to methicillin. Data collected allow to determine whether the deposited and processed municipal waste contains multidrug-resistant strains of staphylococci that may pose a threat to people having contact with them and, in a broader context, to public health.

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION OF *Staphylococcus aureus*

Waste samples used in this study were obtained in the process of mechanical treatment in a mechanical-biological treatment plant in Kraków, Poland (50.032445247N, 20.061035156E). Samples (n = 10) of municipal waste were collected representatively from a pile (located in covered and lockable warehouse), from a depth of approx. 1 m. Each sample (Photo 1) was collected to a sterile 1,000 cm^3 container and immediately transported to the laboratory for microbiological analyses. Isolation of microorganisms from the waste was carried out by Koch's serial dilution method [Pawłat *et al.* 2021]. Chapman agar medium (Biocorp, Poland) was used and cultures were incubated at 37°C for 24 h. After incubation, milky bacterial colonies, grown on Chapman agar (Biocorp, Poland), were used for identification. Identification started by making Gram stained microscopic preparations. Colonies, which in the microscopic preparation were identified as

Photo 1. Sample of municipal waste used in the experiment (phot. *K. Wolny-Koładka*)

Gram-positive cocci, arranged in characteristic clusters, were submitted for further species identification using the MALDI-TOF MS technique (Bruker Daltonik, Germany). Species identification using the MALDI-TOF MS technique was performed according to the methodology recommended by the manufacturer and guidelines provided in publications by other authors [Kosikowska *et al.* 2015, Kozdrój *et al.* 2019].

DRUG RESISTANCE DETERMINED BY THE DISK DIFFUSION METHOD

The disk diffusion method recommended by the European Committee on Antimicrobial Susceptibility [EUCAST 2021] was used to determine the antibiotic resistance of staphylococci. Single bacterial colonies from 18-hour, pure S. *aureus* cultures, were collected with a sterile swab and placed in test tubes with saline solution (0.9% NaCl), vortexed, and adjusted to a concentration of 0.5 McFarland using a densitometer (DEN-1, Biosan, Poland). A sterile swab was immersed in the suspension and the inoculum was evenly spread over the previously prepared Petri dishes with MHA medium (Mueller-Hinton agar, Biocorp, Poland). Sterile antibiotic disks (Oxoid, Ireland) were applied to the cultures. The cultures were incubated at 37°C for 18–24 h, after which zones of growth inhibition (mm) around the discs were read [EUCAST 2021]. The following reference strains were used as a quality control for the disk diffusion method: *S. aureus* ATCC 25923 (methicillin-sensitive) and *S. aureus* ATCC 43300 (methicillin-resistant) [Wolny-Koładka 2018].

DNA ISOLATION AND DETECTION OF *mecA* GENE

DNA was extracted from pure staphylococci cultures with the use of the Genomic Mini Kit (A&A Biotechnology, Poland), in accordance with the manufacturer's instructions. The primers *mecA*-F (5'-GTAGAAATGACTGAACGTCCGATAA-3') and *mecA*-R (5'-CCAATTCCACATTGTTTCGGTCTAA-3') were used for PCR reactions, 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 whose volume totaled to 25 mm^3. DNA amplification was performed with temperature profile as follows: initial denaturation at 94°C for 2 min followed by 30 amplification cycles (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 carried out with the use of a T100TM Thermal Cycler (Bio-Rad, the USA). The PCR products were electrophoresed for 40 min on a 1% SimplySafe (EurX) agarose stained gel in 1×TBE. They were then visualised under UV light and documented using the Gel Doc (Bio-Rad) system. The length of the analysed PCR products was assessed using the GeneRulerTM DNA Ladder Mix, 100–1000 bp (Thermo Scientific) [Wolny-Koładka 2018].

RESULTS AND DISCUSSION

The isolation of staphylococci from municipal waste has allowed to collect 250 strains identified as *S. aureus*. Staphylococci can be isolated from water, soil, air and dust. They are also a natural ingredient of human and animal microflora [Lenart-Boroń *et. al.* 2017]. Hence, their presence in municipal waste should not be surprising. The high number and relatively high survival of staphylococci in waste can be undoubtedly attributed to the morphological properties of waste, i.e. its structure and high porosity. Due to its heterogeneous and very diverse composition (mixed municipal waste containing plastic and cellulose elements as well as plant biomass, etc.), structure with different absorption and ability to accumulate water, waste is a very friendly environment for microorganisms [Wolny-Koładka *et al.* 2020; Wolny-Koładka, Żukowski 2019]. Such conditions provide a kind of protection against unfavourable weather conditions (temperature, wind, rainfall) which extends the life of microorganisms [Zhou, Wang 2013].

Results of phenotypic analyses regarding the drug resistance profile are shown in Table 1. *S. aureus* isolates most frequently presented resistance to cefoxitin (24%), erythromycin (7.2%) and tetracycline (6.0%). No resistance to mupirocin was found among the collected staphylococci. To the best of our knowledge, the literature lacks information on the presence of drug-resistant *S. aureus* strains in municipal waste. Therefore, the analyses carried out in this study are extremely interesting and valuable from the cognitive point of view and bring a lot of key information on hitherto unrecognised microbiological threats to human health. Given the fact that no studies by other authors are available to confront data collected, the results were referred to publications describing the presence of drug-resistant staphylococci in municipal, agricultural and hospital wastewater. Goldstein *et al.* [2012] reported that *S. aureus* strains resistant to, inter alia, erythromycin, tetramycin, clindamycin, ciprofloxacin, tri-

Table 1. Frequency (%) of antimicrobial resistance in *Staphylococcus aureus* strains isolated from waste

Antimicrobial (symbol, µg)	Breakpoint values (mm) [EUCAST 2021] S ≥ / R <	Number of isolates	Share of isolates in the total number of strains (%)
Total		**250**	**100**
Resistant strains		**115**	**46**
Cefoxitin (FOX, 30)	22	**60**	24
Ciprofloxacin (CIP, 5)	21	3	1.2
Doxycycline (DO, 30)	22/19	5	2.0
Erythromycin (E, 15)	21/18	**18**	7.2
Gentamicin (CN, 10)	18	2	0.8
Clindamycin (DA, 2)	22/19	7	2.8
Mupirocin (MUP, 200)	30/18	0	0
Norfloxacin (NOR, 10)	17	1	0.4
Tetracycline (TE, 30)	22/19	**15**	6.0
Trimethoprim/sulfamethoxazole (SXT, 25)	17/14	4	1.6
mecA gene		180	72

Explanations: values ≥10 are in bold type; S ≥ susceptible strain, R < resistant strain.
Source: own study.

methoprim/sulfamethoxazole and gentamicin were present in wastewater samples they tested. In the same vein, strains insensitive to the above-mentioned antibiotics were found in our study. The wastewater tested by Goldstein *et al.* [2012] came from several treatment plants to which water was discharged from households, food production, and agricultural areas. Similar results were obtained by Börjesson *et al.* [2010] who also investigated the presence of MRSA in municipal wastewater. Resistance to β-lactams was demonstrated in 65% of *S. aureus* isolates; moreover, multidrug-resistant strains were identified in activated sludge and inlet water, 40% and 21% respectively. The authors showed the presence of staphylococci resistant to, among others, cefoxitin (MRSA), erythromycin, clindamycin, ciprofloxacin, trimepthoprin and tetracycline [Börjesson *et al.* 2010]. In the same vein, our study showed that the most abundant were strains resistant to cefoxitin, erythromycin and tetracycline. Already in their earlier study, Börjesson *et al.* [2009] found MRSA in samples collected from a municipal sewage treatment plant. Shukla and Sahu [2021] isolated bacteria from industrial wastewater, including 31% of methicillin-resistant strains of *S. aureus*. Boopathy [2017] analysed the presence of *S. aureus* in a small, rural wastewater treatment plant. In that study, methicillin-resistant staphylococci were detected in both raw and treated wastewater. This is particularly worrying, as it indicates that wastewater treatment plants do not capture this type of pollution [Boopathy 2017].

Particularly noteworthy is the great discrepancy in results concerning methicillin resistance of staphylococci. Following the disk diffusion test, the resistance to cefoxitin was found in 60 (24%) strains, whereas genetic analyses allowed to detect the *mecA* gene in as many as 180 (72%) isolates, which were regarded as methicillin-resistant. The large disproportions in results acquired by genetic and phenotypic methods prove that classical methods used to detect resistance to methicillin are very imprecise. On the other hand, methods based on genotyping detect methicillin resistance with a very high sensitivity and therefore should be considered repeatable and reliable [Wolny-Koładka *et al.* 2014]. As shown in our previous study [Wolny-Koładka 2018] and in the study by other authors [Karakulska *et al.* 2012], the *mecA* gene was detected both in cefoxitin-resistant isolates, but also in those classified as methicillin-sensitive by the disk diffusion test. Gomez *et al.* [2016] obtained similar results relating to the presence of drug-resistant *S. aureus* strains in urban wastewater treatment plants. The *S. aureus* strains collected in their study, which had the *mecA* gene, presented both resistance and sensitivity to methicillin in the phenotypic test [Gomez *et al.* 2016]. Given the increasing drug resistance, extremely worrying are the results presented by Börjesson *et al.* [2010] regarding the presence of the *mecA* gene directly in both hospital and municipal wastewater. This gene has been isolated directly from wastewater, which clearly shows that even when the pathogenic bacterium is eliminated, the genetic material still represents a biological risk [Börjesson *et al.* 2010]. Makowska *et al.* [2021] found genes encoding resistance to β-lactam antibiotics in the genomes of bacteria isolated from different stages of wastewater treatment. The *mecA* gene in the genomes of *S. aureus* has been noted at every stage of wastewater treatment [Makowska *et al.* 2021]. Moreover, methicillin-resistant staphylococci carrying the *mecA* gene have also been isolated from employees of the municipal sewage treatment plant [Zieliński *et al.* 2020]. Boopathy [2017] confirmed the presence of the *mecA* gene in both *S. aureus* strains isolated from municipal sewage and a free gene floating in wastewater. Antibiotic resistance can therefore be transferred not only between bacteria through genetic transformation, but also through free genes in the environment, and consequently, they can spread throughout the ecosystem, posing a serious threat to public health [Boopathy 2017].

S. aureus most often showed resistance to 1, 2, 3 or 4 antibiotics at the same time. Similar results were obtained for MRSA isolates from municipal wastewater [Börjesson *et al.* 2010]. MDR strains accounted for 18% (45 isolates), which, compared to studies by other authors, should be considered a rather low share (Tab. 2). Among staphylococcal strains isolated from wastewater by Börjesson *et al.* [2010], 40% were classified as MDR. In the study by Goldstein *et al.* [2012], the share of MDR bacteria among methicillin-resistant *S. aureus* was 93%.

Table 2. Number of MDR and *Staphylococcus aureus* strains resistant to different antimicrobials

Specification	Number of antibiotics											MDR
	0	1	2	3	4	5	6	7	8	9	10	
***S. aureus* (*n* = 250)**	135	30	27	20	16	0	9	0	4	6	3	45
Share (%) of isolates	54.0	12.0	10.8	8.0	6.4	0	3.6	0	1.6	2.4	1.2	18.0

Explanations: MDR = multidrug-resistant, *n* = number of isolates.
Source: own study.

CONCLUSIONS

The study allowed to isolate, identify and assess the drug resistance profile of 250 isolates of *S. aureus* from mixed municipal waste. According to the assumed hypothesis, many of the collected strains were antibiotic-resistant, and the study also found bacteria with the MDR phenotype. Methicillin resistance of the isolates was found in the disk diffusion test and by detecting the *mecA* gene responsible for its formation. The results of this study indicate that municipal waste is a considerable source of drug-resistant staphylococci. It should be remembered that all *S. aureus* strains originated from municipal waste and were closely related to human activity. Waste examined was neither medical nor hazardous. The presence of methicillin-resistant *S. aureus* in waste may pose a threat to people having contact with such waste and, in a broader context, to public health. In conclusion, it is justified to conduct further research to identify risk factors for the spread of drug resistance through

municipal waste, landfills, and waste treatment companies. This is particularly important because municipal waste, due to its diverse and time-dependent composition, constitutes a reservoir of drug-resistant strains which can spread in the environment and pose a threat from an epidemiological point of view.

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