Aminoglycosides resistance in clinical isolates of *Staphylococcus aureus* from a University Hospital in Bialystok, Poland

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Abstract: *Staphylococcus aureus* obtained from a University Hospital in Poland were characterized in relation to resistance to aminoglycoside antibiotics and the distribution of the genes encoding the most clinically relevant aminoglycoside modifying enzymes (AMEs). Of a total of 118 *S. aureus*, 45 (38.1%) isolates were found to be resistant to at least one of the tested antibiotics. All aminoglycoside resistant isolates except one 44 (97.8%) were resistant to kanamycin. The majority of strains 37 (82.2%) and 32 (71.1%) expressed resistance to neomycin and tobramycin, respectively. Eleven strains (24.4%) were resistant to gentamicin or amikacin. All *S. aureus* strains were sensitive to netilmicin. The most prevalent resistance gene was *aac(6')-Ie+aph(2')* found in 13 (28.9%) strains and 12 (26.7%) isolates carried *ant(4')-Ia* gene, whilst *aph(3')-IIIa* gene was detected in only 7 (15.6%) isolates. Additionally, the *ant(6)-Ia* and *str* genes were detected in 14 (31.1%) and 2 (4.4%) strains, respectively. Ten (22.2%) strains resistant to amikacin, tobramycin, kanamycin or neomycin did not harbor any of the above-noted genes.

Key words: Aminoglycoside - Aminoglycoside modifying enzymes (AME) - Streptomycin - *Staphlococcus aureus*

Introduction

Staphylococcus aureus is a major cause of hospital- and community-acquired infections, and can results in serious consequences. Hospital infections caused by *S. aureus* include those affecting the bloodstream, lower respiratory tract, skin and soft tissues, as well as ventilator-assisted pneumonia and central venous catheter-associated bacteraemia. The importance of *S. aureus* as a human pathogen, apart from its ability to cause a diverse range of life-threatening infections, is its extraordinary potential to develop antimicrobial resistance [7].

One of the class of antibiotics playing an important role in the therapy of serious staphylococcal infections are aminoglycosides despite reports of increased resistance to these drug in many countries of the Europe [11]. The main mechanism of aminoglycoside resistance is drug inactivation by aminoglycoside-modifying enzymes (AMEs) encoded within mobile genetic elements [14]. The following three AMEs are of particular significance among staphylococci since they modify and thereby inactivate the traditional aminoglycosides of therapeutic importance: aminoglycoside-6'-N-acetyltransferase/2"-O-phosphoryltransferase [AAC(6')/APH(2")], aminoglycoside-4'-O-nucleotidyltransferase I [ANT(4')-I] and aminoglycoside-3'-O- phosphoryltransferase III [APH(3')-III]. Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci are mediated by bifunctional enzyme displaying AAC(6') and APH(2") activity encoded by *aac(6')-Ie+aph(2")* gene. Resistance to neomycin, kanamycin, tobramycin and amikacin is mediated by an ANT(4')-I enzyme encoded by *ant(4')-Ia* gene. The APH(3')-III enzyme, which inactivates kanamycin and neomycin is encoded by *aph(3')-IIIa* gene [12, 14]. Resistance to strepto-

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mycin in staphylococci is associated with the enzymes ANT(6)-I and APH(6)-I encoded by *ant(6)-Ia* and *str* genes, respectively [11,12,14].

As aminoglycosides resistance and the distribution of the genes encoding aminoglycoside-modifying enzymes has not been well characterized or documented in large collection of *S. aureus* in Poland, we investigated *S. aureus* strains isolated from a University Hospital, in order to gain some insight into the nature of the resistance to this class of antibiotics.

Materials and methods

Bacteria. A total of 118 *S. aureus* isolates obtained during 2002-06 at the University Hospital were included in this study. All isolates were identified as *S. aureus* by ID 32 Staph (bioMerieux, France) according to the manufacturer instructions. Pure culture were preserved in 30% glycerol at -80°C and were subcultured in brain heart infusion broth, and incubated at 37°C prior to further testing. Multiple isolates of the same patient were excluded.

Antimicrobial agents and susceptibility testing. Antimicrobial susceptibility testing was performed using disk diffusion method on Mueller-Hinton agar plates. *S. aureus* ATCC 29213 was used as quality control strain for *in vitro* susceptibility testing. The antibacterial agents tested were: gentamicin (10 µg), amikacin (30 µg), netilmicin (30 µg), tobramycin (10 µg), kanamycin (30 µg) and neomycin (30 µg). Susceptibility to aminoglycosides was interpreted according to document M2-A8 of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards , NCCLS) [9].

PCR amplification of aminoglycosides resistance genes. All isolates demonstrating resistance to at least one of the aminoglycoside antibiotics were screened for the presence of the *aac(6')-Ie/aph(2'')*, *ant(4')-Ia*, *aph(3')-IIIa*, *ant(6)-Ia* and *str* genes. Genomic DNA as a template for PCR assay was extracted by incubating with lysostaphin followed by purification with a commercially available purification kit (Eurx, Poland).

The *aac(6')-Ie/aph(2'')*, *ant(4')-Ia*, *aph(3')-IIIa*, *ant(6)-Ia* and *str* genes were detected by previously described primers [2, 5, 11]. PCR was performed in a Perkin-Elmer DNA thermal cycler, for the first of fourth genes at 94°C for 10 min, then 35 cycles at 94°C for 45 s, 60°C for 60 s, and 72°C for 60 s. A final extension cycle of 72°C for 5 min was applied. The following program was used for amplification of fragment of *str* gene: denaturation at 94°C for 3 min, then 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s, and final extension at 72°C for 10 min.

The Gene Ruler 100 bp DNA Ladder Plus (MBI Fermentas, Lithuania) was used as a molecular size marker on the gels. All isolates were tested at least twice at independent occasions before being considered as positive.

Results

Of total 118 *S. aureus* isolates included in this study, 45 (38.1%) were resistant to at least one of the tested aminoglycoside antibiotics (Table 1). All isolates except one (97.8%) were resistant to kanamycin. The majority of strains 37 (82.2%) and 32 (71.1%) expressed resistance to neomycin and tobramycin, respectively. Eleven strains (24.4%) were resistant to gentamicin or amikacin. The most active antimicrobial

agents against *S. aureus* was netilmicin. All isolates were sensitive to this drug.

With regard to resistance phenotypes, only 3 (6.7%) isolates were resistant to the greatest number of antibiotics tested, and were sensitive only to netilmicin. The great number of *S. aureus* isolates 13 (28.9%) were resistant to tobramycin, kanamycin and neomycin followed by resistance to kanamycin and neomycin in 12 (26.7%) isolates. Resistance to amikacin, tobramycin, kanamycin and neomycin was observed in 7 (15.6%) isolates, and the same number of strains were resistant to gentamicin, tobramicin and kanamicin. The other three resistance phenotypes: gentamicin/tobramycin/kanamycin/neomycin, amikacin/kanamycin/neomycin or tobramycin were observed in one (2.2%) isolate.

All of aminoglycosides resistant *S. aureus* were screened for the presence of three genes encoding the most clinically relevant aminoglycoside modifying enzymes (Table 1). The most prevalent resistance gene was *aac(6')-Ie+aph(2')* found in 13 (28.9%) strains. Twelve (26.7%) isolates carried *ant(4')-Ia* gene, whilst *aph(3')-IIIa* gene was detected in only 7 (15.6%) isolates. Additionally, the *ant(6)-Ia* and *str* genes were detected in 14 (31.1%) and 2 (4.4%) strains, respectively. Ten (22.2%) strains resistant to amikacin, tobramycin, kanamycin or neomycin did not harbor any of the above-noted genes.

Discussion

Aminoglycoside resistance is common in *S. aureus* isolated from different countries, and especially gentamicin resistance, is of clinical importance because it can compromise the therapeutic effectiveness of these antibacterial agents [15].

Since PCR is a reliable tool for the identification of aminoglycoside modifying enzyme gene in staphylococci [5,11], it was used in this study to detect the *aac(6')-Ie+aph(2')*, *ant(4')-Ia*, *aph(3')-IIIa*, *ant(6)-Ia* and *str* genes in the *S. aureus* tested, and, hence the enzymes they encode.

The incidence of ANT(4') in this study was higher than that reported in other studies were the AAC(6')-APH(2'') enzyme has been found to be the most common AME in *S. aureus* [3,10,15]. The higher incidence of the ANT(4') enzyme was because it included isolates that were resistant to kanamycin, gentamicin susceptible and contained genes for only the ANT(4') enzyme. The results were in agreement with results of antibiotic resistance testing which demonstrated that 44 of the 45 isolates were kanamycin resistant, and with results aminoglycoside resistance in *S. aureus* isolated in Kuwait hospitals [13]. Similarly, the study carried out in Japan reported much higher prevalence of ANT(4') enzyme than that of the other two AME

Table 1. Aminoglycoside resistance genes in *S. aureus* with different aminoglycoside resistance phenotypes.

Resistance phenotypes[a]	AMEs	No. of isolates
Gm, Ak, Tob, K, N	AAC(6')-APH(2")+APH(3')+ANT(6)	1
Gm, Ak, Tob, K, N	AAC(6')-APH(2")+ANT(4')	1
Gm, Ak, Tob, K, N	AAC(6')-APH(2")	1
Gm, Tob, K, N	AAC(6')-APH(2")+ ANT(6)	1
Gm, Tob, K	AAC(6')-APH(2")	6
Gm, Tob, K	AAC(6')-APH(2")+APH(6)	1
Ak, Tob, K, N	ANT(4')	5
Ak, Tob, K, N	-	2
Ak, K, N	ANT(6)	1
Tob, K, N	AAC(6')-APH(2")+ANT(4')	1
Tob, K, N	AAC(6')-APH(2")+ANT(6)+APH(6)	1
Tob, K, N	ANT(4')	4
Tob, K, N	-	7
K, N	APH(3')	1
K, N	APH(3')+ANT(6)	5
K, N	-	1
K, N	ANT(6)	5
Tob	ANT(4')	1

Abbreviations: [a]Gm, gentamicin; Ak, amikacin; Tob, tobramycin; K, kanamycin; N, neomycin.

enzymes [6]. However, insignificant frequently was detected genes for AAC(6')-APH(2") enzyme, which is similar to other reports that studies *S. aureus* [4,8, 10,12,15,16]. Although, it have been demonstrated an upward in the proportions of AAC(6')-APH(2") and ANT(4') in aminoglycoside resistant *S. aureus* in our study in comparison with other European hospitals [11]. In contrast, netilmicin had excellent activity against all isolates. Similarly, Vanhoof *et al.* [15] reported a low incidence of netilmicin resistance among *S. aureus* in Belgium hospitals. Because of its activity against the all isolates, a combination of netilmicin and tobramycin has been advocated for the treatment of infections caused by aminoglycoside resistant strains producing AAC(6')-APH(2") enzyme since the combination acts synergistically [1].

Streptomycin resistance is mainly associated with *ant(6)-Ia, str*, or ant(3")-Ia genes [14]. Within the *S. aureus* population under study, resistance to this antibiotic was mainly encoded by *ant(6)-Ia*. Comparative analysis of our results is difficult due to poorly data on resistance to streptomycin in *S. aureus*.

The strains harboring *aac(6')-Ie+aph(2')* gene encoding AAC(6')-APH(2") enzyme are considered to be resistant to gentamicin and even to all aminoglycosides. However, in our study this gene was detected in two gentamicin susceptible isolates. In some studies have also reported similar findings [10, 13, 15]. The detection of resistance genes in antibiotic susceptible isolates may be due to the amplification of repressed antibiotic resistance gene [10] or AME of these strains display lower enzymatic activity, detected also in other study of *S. aureus* [15].

It is noteworthy to mention that in the 10 isolates demonstrating phenotypic resistance to any one of the antibiotics tested, we did not detect a known gene encoding an aminoglycoside modifying enzyme which could account for the phenotype. Because it is possible to deduce the type of enzyme present from the patterns of resistance (resistance to amikacin, tobramycin, kanamycin and neomycin, and susceptibility to gentamicin), these strains pointed toward the presence of the enzymes encoded by *ant(4')-Ia* or *aph(3')-IIIa* genes. The failure to detect these two genes may be due to either the presence of an *ant(4')-Ia* or *aph(3')-IIIa* variant gene that cannot be detected with the primers used or this suggests that new aminoglycoside resistance genes are circulating within the *S. aureus* population.

DNA methods that detect resistance genes appear to be very sensitive in detecting resistance mechanism even when the resistance is not expressed. This is significant because exposure of these organism to the antibiotics at a later date will result in the expression of full resistance if the genes are present, and can influence proper prescription, and use of appropriate agents for therapy.

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