THE RELATION OF METHICILLIN-RESISTANCE TO GROWTH TIME IN STAPHYLOCOCCI

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ABSTRACT

Background*: With the biochemical and molecular studies on penicillin-binding proteins associated with methicillin-resistance in staphylococci the determination and interpretation of the resistance have been changed. Particularly careful evaluation of staphylococci isolated from blood cultures before instituting therapy is of utmost significance in regard to morbidity and mortality. One of the important criteria of this evaluation is the growth time of staphylococci. The purpose of this study was to determine the relation of methicillin-resistance to growth time in Staphylococcus aureus and methicillin-resistant staphylococci.*

Methods*: The study was carried on a total of 66 clinical isolates of staphylococci including 18 methicillin-susceptible Staphylococcus aureus (MSSA), 14 methicillin-resistant Staphylococcus aureus (MRSA), 19 methicillin-susceptible coagulase-negative staphylococci (MSCNS) and 15 methicillin-resistant coagulase-negative staphylococci (MRCNS). Following growth on subculture under similar conditions, all isolates were inoculated in equal quantities into hemoculture bottles, the optimum time-of-positivity in minutes for each isolate was calculated, and the growth times of the 4 isolate groups were statistically compared.*

Results*: The longest growth time was detected in MRCNS. The growth time was longer in MRSA and MRCNS than in MSSA and MSCNS, respectively; however, a statistically significant difference was observed between MRCNS and MSCNS isolates.*

Conclusion*: In conclusion, it is known that in methicillin resistant Staphylococcus aerus, PBP2a which is responsible for methicillin resistance takes over the role penicillin-binding protein of PBP1, PBP2 and PBP3 in peptidoglycan synthesis. But, other possible factors affecting the resistance mechanisms or growth time of MRCNS should be investigated.*

Key words*: Staphylococcus aureus, coagulase-negative staphylococci, methicillin-resistance, growth time.*

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Introduction

Treatment options are quite limited in infections due to methicillin-resistant staphylococci. The methicillin-resistance particularly in blood stream infections is a significant cause of morbidity and mortality[(1)]. The correct evaluation of blood cultures performed for the detection of blood stream infections is one of the main challenges in the clinical microbiology laboratory. Early and accurate identification of blood isolates and subsequent antibiotic susceptibility tests are crucial to enable optimal targeted antimicrobial therapy and to decrease the associated mortality. Although recently molecular techniques such as nucleic acid probes and polymerase chain reaction (PCR) have been developed for rapid diagnosis, still the most sensitive and reliable method of detecting blood stream infections is hemoculture[(2,3)]. Full-automatic systems for hemoculture are now increasingly used in Turkey. Although these systems shorten the bacterial reproduction time, because of their sensitivity and rich medium content, contaminations are frequently encountered when prior skin antisepsis and prior disinfection of the bottle caps are omitted[(4)]. Methicillin-resistant staphylococcal colonization increases in long-hospitalized, especially in intensive care patients. In such patients blood culturing without prior skin cleaning easily leads to the contamination of hemoculture bottles with methicillin-

resistant staphylococci, a situation which causes interpretation difficulties in the clinical microbiology laboratory especially when the hemoculture is not repeated. It is evident that reporting the results of such specimens is not an easy task when factors affecting the time of reporting are considered, i.e. kind of microorganism in the specimen, microbial bioburden of the specimen, variations in microbial reproduction times and time span the specimen is left at room temperature. In our previous study[5], we had found a significant difference between the growth times of causative agents and contaminants in hemocultures, a result in agreement with the literature. However, in the same study we had also found that, as infectious agents or contaminants, all methicillin-resistant *Staphylococcus aureus* (MRSA) isolates required a longer time for growth than methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates. The MRSA isolates grew in an average of 26 hours whereas MSSA isolates in 11 hours, and the difference in between was statistically significant ($p< 0.01$). This situation indicates the need for a different interpretation of growth time of staphylococci in blood cultures.

The purpose of this study was to determine the relation of methicillin-resistance to growth time in methicillin-susceptible and methicillin-resistant staphylococci.

Materials and methods

The study included 66 clinical strains of staphylococci isolated in the Clinical Microbiology Laboratories of Medical Schools of Namık Kemal University (N.K.Ü.), Karadeniz Technical University and Abant Izzet Baysal University in 2013. The strains which were isolated from hemoculture have been identified and then, they were sent to N.K.Ü. Clinical Microbiology Laborotory in transport medium for the experiments. The material included 18 MSSA, 14 MRSA, 19 methicillin-susceptible coagulase-negative staphylococci (MSCNS) and 15 methicillin-resistant coagulase-negative staphylococci (MRCNS). As controls ATCC 43300 (MRSA) and ATCC 25923 (MSSA) were used.

In N.K.Ü. Microbiology Laborotory, the identification of the strains were confirmed before the experimental studies. For the identification of staphylococcal isolates; gram-staining, catalase test, tube-coagulase test, and semi-automatic identification system BBL Crystal (BD BBL Crystal Identification Systems, Gram-Positive ID Kit, Becton Dickenson, USA) were used. The methicillin-resistance of the isolates was determined by using cefoxitin (30 μg, BD, USA) and oxacillin (1 μg, BD, USA) discs. The Kirby-Bauer disc diffusion method with cefoxitin and oxacillin discs was performed according to the recommendations of CLSI[6].

To compare the growth times of methicillin-susceptible and methicillin-resistant isolates a hemoculture system (BACTEC 9050, Becton Dickinson, USA) was used. The isolates were streaked on agar with 5% sheep blood as to obtain single colonies. After overnight incubation of the inoculated plates at 35° C, equal-size colonies were selected, and a single colony from each culture plate was inoculated into 5 ml of Todd-Hewith broth (THB). Following 1-hour incubation at 35° C, after arranged to 0.5 Mac Farland turbidity, each culture in THB was diluted 10-fold 4 times as to obtain a final dilution of 1:10.000, and 5 ml of the diluted bacterial suspension was inoculated into pediatric hemoculture bottles containing 45 ml liquid medium. Additionally, with the purpose of controlling purity and standardizing the bacterial count, 100 μl of the dilutions of each isolate was streaked on agar with 5% sheep blood for plate-counting.

All hemoculture bottles were concurrently placed in the hemoculture system. Upon positive signal growth report was obtained from the system and time span in minutes for growth in each bottle was calculated.

For data access and analysis PASW Statistics 18 for Window Statistics Package Program was used. The normality of the groups was tested by using the Kolmogorov-Smirnov test. For the comparison of two groups, among normally distributed groups independent sample t test and among abnormally distributed groups Mann-Whitney U test were performed. The value of $p<0.05$ was accepted as statistically significant.

Results

Among the staphylococcal isolates 38, 15 and 13 were isolated in various specimens of patients hospitalized in the hospitals of Abant Izzet Baysal University, Karadeniz Technical University and Namık Kemal University, respectively. The shortest time-of-positivity of growth of 450 minutes was detected in one MSSA isolate and the longest time-of-positivity of 970 minutes in one MRCNS isolate.

The average time-of-positivity for MSSA, MRSA, MSCNS and MRCNS isolates was calculated as 618.33, 642.86, 721.58 and 845.33 minutes, respectively. The average time-of-positivity and p value for each staphylococcal group are shown in the Table 1.

Group	Number	Average Growth Time (hours)	Standart Deviation	p value
MSSA	18	618.33	280.50	0.53
MRSA	14	642.86	247.50	
MSCNS	15	845.33	204.86	0.046
MRCNS	19	721.58	143.38	

Table 1: Data of groups and their statistical results.
MSSA: Methicillin-susceptible Staphylococcus aureus
MRSA: Methicillin-resistant Staphylococcus aureus
MSCNS: Methicillin-susceptible coagulase-negative staphylococci
MRCNS: Methicillin-resistant coagulase-negative staphylococci

Discussion

Shortly after the introduction of methicillin into clinical use in 1960, methicillin-resistance in staphylococci was reported (1961). The methicillin-resistance in staphylococci is not acquired by mutations of existing penicillin-binding protein (PBP) genes, but by acquiring new PBP genes.

Penicillin-binding proteins are cytoplasmic membrane proteins; all β-lactam antibiotics bind to PBPs, which are essential for bacterial cell wall biogenesis[7]. In *Staphylococcus aureus* (*S. aureus*) 4 types of PBP have been described. Of these PBP2 and PBP3 are the major PBPs, both showing peptidoglycan transpeptidase activity; PBP4 is not an indispensable PBP, but exerts DD-carboxypeptidase, transpeptidase and β-lactamase effect and plays a role in the formation of cross-linking of peptidoglycan chains[8]. Penicillins and cephalosporins affect *S. aureus* by binding both to PBP2 and PBP3. The exceptions are cephalexin, cefradin and cefaclor found in the same cephalosporin group, which intensely bind to PBP3 and cefotaxim which selectively binds to PBP2. Studies on the effect of these two groups of antibiotics on the cell wall synthesis in staphylococci have revealed that PBP2 is the major peptidoglycan transpeptidase and that PBP3 is the transpeptidase in cell-division and the major PBP determining antibiotic activity[9]. In methicillin-resistant staphylococci the newly synthesized PBP2a and PBP2' are coded by the *mecA* gene in the SCCmec. Although PBP2a and PBP2' show low affinity to all β-lactam antibiotics including the new cephalosporins and carbapenems, they take over the responsibilities of PBP1, PBP2 and PBP3 in terms of transpeptidation in cell wall synthesis[10].

There has been a great deal of biochemical and molecular research in the last 40 years into PBPs because of their role in bacterial reproduction. The PBPs are broadly divided into high-molecular-weight and low-molecular-weight categories and into 3 classes of A, B and C. Each bacterium has a specific PBP designation, however, some bacteria show similar effect as in the example of *S. aureus* PBP3 and E. coli PBP' where PBP3 and PBP' share a similar structure[11]. High molecular weight PBPs are the targets for β-lactam antibiotics and play a critical role in cell reproduction. *S. aureus* holds 4 PBPs, namely, PBP2 in Class A, PBP1 and PBP3 in Class B, and PBP4 in Class C. In *S. aureus* susceptible to β-lactam antibiotics there are 2 of Class B PBPs (1 and 3) whereas in *S. aureus* resistant to β-lactam antibiotics there is additionally PBP2a which shows low β-lactam affinity. The transglycolization and transpeptidation activities of PBP2a seem to be similar to those of other PBPs.

In our former study[5], the growth times of bacterial agents had been compared with those of contaminants in hemocultures. Since the numbers of contaminants inoculated are fewer than the numbers of agent bacteria at the time of inoculation with consequent longer time-to-positivity, in the present study we standardized the number of bacteria inoculated and determined the changes in time-to-positivity. Moreover, in our former study[5] all patients suspected of bacteremia and from whom blood specimens were obtained had been already under antibacterial therapy; in view of this situation, in the present study we used stock-culture bacteria in place of primary blood specimens. All bacteria used were formerly grown under similar conditions and then inoculated in equal quantities into hemoculture bottles. Thus, we tried to eliminate most of the factors that can affect the bacterial growth time and paid attention that no difference existed between the groups of bacteria except methicillin-resistance and coagulase-reaction.

We did not use molecular methods in assessing methicillin-resistance in staphylococci and did not perform the *mecA* gene test. However, we

simultaneously evaluated the oxacillin and cefoxitin discs. The sensitivity and specificity of oxacillin and cefoxitin disc diffusion test used in assessing meticilin-resistance have been reported to be between 98 and 100%[12-14].

In this study on the relation of methicillin-resistance to growth time in staphylococci, in coagulase-negative staphylococci the relation between the methicillin-resistance and growth time was found statistically significant. Although among *S. aureus* isolates the average growth time was calculated to be longer in MRSA than in MSSA, the difference between the two groups was statistically insignificant. All studies in the literature on PBPs and peptidoglycan biosynthesis are biochemical and molecular investigations on the structure of PBPs in staphylococci[11]. In the light of the obtained data, the key determinant of the β-lactam resistance in MRSA strains is PBP2a. Because of its low affinity for β-lactams, PBP2a provides the transpeptidase and transglycolization activities of PBP1, PBP2 and PBP3 normally produced by *S. aureus*. These results suggest that in coagulase-negative staphylococci other mechanisms might be responsible for the function of PBPs in peptidoglycan synthesis.

Conclusions

There is a statistically significant relation between methicillin-resistance and growth time in coagulase-negative staphylococci. Further investigations are required about how methicillin-resistance and acquired PBP2a affect the growth physiology, primarily the peptidoglycan synthesis, in these bacteria.

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