Influence of decreased penicillin susceptibility on growth rate of beta haemolytic streptococci

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**Background & objectives:** Beta haemolytic streptococci (BHS), especially group A are still highly susceptible to penicillin. One possible explanation for this could be reduced growth capability in penicillin resistant BHS mutants. The present study was therefore undertaken to analyze the growth rates of BHS with decreased susceptibility to penicillin.

**Methods:** Serial passages in the medium with subinhibitory concentration of penicillin were done to induce resistance to this antibiotic in 12 clinical isolates of BHS serogroups A, B, C, and G. Both penicillin susceptible (parental) and variants with decreased susceptibility to penicillin (laboratory strains) were grown in three different media and their growth rates were determined by counting the number of bacterial colonies and by measuring optical density of bacterial culture.

**Results:** The lowest increase in minimal inhibitory concentration (MIC) value for penicillin (8-16 times) was obtained in BHS group A isolates, while the increase in MIC values of BHS groups B, C and G strains was higher (64-128 times) and they reached the level of complete resistance. Laboratory variants differed significantly from parental in their morphological and cultural characteristics. There were no statistically significant differences between the growth rates of penicillin susceptible and variants with decreased susceptibility to penicillin, though a delay in multiplication of the laboratory strains during exponential phase of growth was noted.

**Interpratation & conclusion:** Though significant differences in phenotypic characteristics of penicillin susceptible and laboratory variants were noted, the results of this study provides no support to the assumption that variants of BHS with decreased susceptibility to penicillin of BHS were incapable for normal growth. Further studies needs to be done to find out the association between the decreased susceptibility to penicillin in the BHS and decreased growth capability in these bacteria.

**Key words** Beta haemolytic streptococci - growth rate - penicillin - resistance - Streptococcus

Since its discovery, though penicillin was extensively used in the treatment of infections caused by streptococci, beta haemolytic streptococci (BHS), especially group A, remained highly susceptible to penicillin. However, failure in the penicillin treatment of infections caused by BHS group A isolates has been reported in up to 30 per cent cases, but no clinical isolate resistant to penicillin has been identified so far. *Streptococcus pyogenes* successfully developed resistance to other antibiotics such as sulfonamides, tetracycline, erythromycin. Also, resistance to penicillin has been reported in other closely related species (*S. pneumoniae*, viridans streptococci, *Enterococcus* spp.)³. It has also been shown that decreased susceptibility to penicillin may be induced in BHS in laboratory conditions. It is generally assumed that resistance to penicillin in BHS, as well as in other streptococci, is not mediated by b-lactamases, but by
low affinity penicillin binding proteins (PBPs)\textsuperscript{5}. PBPs act as enzymes, which catalyze the synthesis of the cell wall. Therefore, it is possible that changes in PBPs in BHS result in development of mutants with severe physiological defects that make them incapable for normal growth. The present study was undertaken with the objective to investigate growth capability of BHS with decreased susceptibility to penicillin.

**Material & Methods**

Among 410 isolates of BHS examined in the laboratory of Institute of Microbiology and Immunology during the period 1999-2001, twelve highly penicillin susceptible isolates of serogroups A, B, C, and G (three strains per serogroup) were selected. Two BHS group A isolates were obtained from throat swabs and one from skin lesion; two BHS group B isolates from vagina and one from urine; groups C and G included one isolate from throat and two from wounds, each.

BHS were identified according to the current recommendation\textsuperscript{6}. Isolates were cultivated in Todd Hewitt broth (THB) and stored at -70°C after adding glycerol to a final concentration of 10 per cent. Minimal inhibitory concentration (MIC) for penicillin (Galenika, Belgrade) was detected by broth dilution test, according to NCCLS\textsuperscript{7} recommendations.

Two groups of BHS were subjected to further analyses - 12 penicillin susceptible clinical isolates (parental) and 12 with decreased susceptibility to penicillin (laboratory strains) that were obtained after 105 serial passages of parental isolates in THB supplemented with subinhibitory concentration of penicillin. Stability of decreased susceptibility to penicillin in laboratory strains was examined by serial sampling of the last subcultures onto the blood agar plates without penicillin. Susceptibility of parental and laboratory strains to beta lactam antibiotics (ampicillin, amoxicillin, cephalexin, cephalor, ceftiraxone) was tested by the agar dilution method according to the NCCLS\textsuperscript{7} recommendations. The morphological and cultural characteristics of pen-s and pen-r strains were compared. Growth rates of the two BHS groups were determined by two different methods. The first one was estimation of number of bacterial colonies on blood agar plates. Each strain of BHS was cultivated in three different media - THB, Brain heart broth (BHB) and THB supplemented with horse serum (THB-HS). Overnight culture of each strain was adjusted to an optical density of 0.6 at 570 nm using an automated Multiskan EX reader (Labsystems, Helsinki, Finland), which corresponds to 3x10^8cfu/ml and further diluted to 10^4cfu/ml. These suspensions were cultivated in THB, BHB, and THB-HS, respectively, at 37 °C for 24 h. The samples (0.1 ml) were plated on the blood agar plates after 2, 4, and 24 h of incubation. The inoculated blood agar plates were incubated for 24 h at 37 °C and the colonies were counted and growth curves were constructed. The second method comprised measuring optical densities of bacterial cultures. Microtiter plates were filled with three different media (THB, BHB, THB-HS). Overnight cultures of each strain were diluted to 10^4cfu/ml and added into the wells (200 µl per well). The plates were incubated for 24 h at 37 °C and optical density was measured at 450 nm using Multiskan EX reader after 2, 4, and 24 h of incubation and growth rate curves were constructed.

Differences in growth rates of parental and laboratory variants were examined by Wilcoxon signed rank test, \( P < 0.05 \) was considered significant.

**Results**

Serial passages of BHS in broth with subinhibitory concentrations of penicillin led to the development of laboratory strains with 8-128 times raised MIC values. The lowest increase in MIC value (8-16 times) was obtained in three BHS group A isolates (Table), while the increase in MIC values of BHS groups B, C and G strains was higher (64-128 times). The increase in the MIC values was discontinuous with periods of raise alternating with periods of stagnation lasting for several passages.

At the end of subcultivation, two group A strains remained susceptible to penicillin (0.125 µg/ml), while the third one reached intermediate level of resistance (0.25µg/ml). Other BHS strains developed complete resistance to penicillin, having MIC equal or above 1µg/ml. Laboratory strains also displayed decreased susceptibility to beta lactam antibiotics other than penicillin (Table). Evaluation of stability of decreased susceptibility to penicillin revealed different patterns in BHS group A.
strains and other BHS strains tested. MIC values of laboratory BHS group A strains were comparable to that of parental isolates after 10 serial passages of the last subculture on the blood agar plates without penicillin. The same procedure resulted in only one to two concentrations decrease in BHS strains belonging to other serogroups.

Comparison of morphological and cultural characteristics between parental and laboratory strains revealed significant differences. While penicillin susceptible isolates expressed all typical features of the particular BHS groups, the cells of laboratory strains were pale in Gram stained smears, their diameters were smaller and chains were longer. Also, the appearance of their colonies was changed. The colonies of laboratory strains were small, white, and, most noticeable, without typical beta haemolysis. All these changes were particularly expressed in strains belonging to groups A and B. However, no differences between parental and laboratory variants were observable after additional 10 passages in medium without penicillin.

Growth rates of penicillin susceptible and those with decreased susceptibility to penicillin were determined by two different methods. Both methods for evaluation of growth rates disclosed similar patterns of growth dynamics in all parental and laboratory variants of BHS strains grown in THB and BHB. The representative growth curves obtained by measuring of optical density of penicillin susceptible and variants with decreased susceptibility to penicillin of one BHS B strain grown in THB are shown in Fig.1. As compared to parental strains, laboratory variants multiplied at slower rate during exponential phase of growth, but there were no significant differences between OD values of parental and laboratory variants after 24 h of incubation. When the strains were grown in media supplemented with horse serum, no delay in growth of variants with decreased susceptibility to penicillin was noted (Fig.2).

### Table: MIC (µg/ml) values for penicillin, ampicillin, amoxyxillin, cephalaxin, cepchachlor and ceftriaxon determined in 12 BHS strains before (parental) and after induction of resistance (laboratory) variants to penicillin

<table>
<thead>
<tr>
<th>BHS groups</th>
<th>Penicillin</th>
<th>Ampicillin</th>
<th>Amoxycillin</th>
<th>Cephalexin</th>
<th>Cephachlor</th>
<th>Ceftriaxon</th>
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<tbody>
<tr>
<td></td>
<td>P</td>
<td>L</td>
<td>P</td>
<td>L</td>
<td>P</td>
<td>L</td>
</tr>
<tr>
<td>A1</td>
<td>0.015</td>
<td>0.125</td>
<td>0.030</td>
<td>0.125</td>
<td>0.030</td>
<td>0.25</td>
</tr>
<tr>
<td>A2</td>
<td>0.015</td>
<td>0.125</td>
<td>0.030</td>
<td>0.125</td>
<td>0.030</td>
<td>0.25</td>
</tr>
<tr>
<td>A3</td>
<td>0.030</td>
<td>0.250</td>
<td>0.015</td>
<td>0.125</td>
<td>0.015</td>
<td>0.25</td>
</tr>
<tr>
<td>B1</td>
<td>0.060</td>
<td>4</td>
<td>0.060</td>
<td>0.5</td>
<td>0.125</td>
<td>1</td>
</tr>
<tr>
<td>B2</td>
<td>0.125</td>
<td>2</td>
<td>0.125</td>
<td>0.25</td>
<td>0.060</td>
<td>1</td>
</tr>
<tr>
<td>B3</td>
<td>0.125</td>
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<td>0.25</td>
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<tr>
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<td>0.5</td>
</tr>
<tr>
<td>G1</td>
<td>0.015</td>
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<td>0.30</td>
<td>0.5</td>
<td>0.030</td>
<td>0.5</td>
</tr>
<tr>
<td>G2</td>
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<td>0.5</td>
<td>0.030</td>
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<tr>
<td>G3</td>
<td>0.030</td>
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<td>0.25</td>
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</table>

P, parental isolates; L, laboratory strains
Since PBPs play a major role in the terminating phase of cell wall synthesis, the changes in these proteins associated with decreased susceptibility to penicillin may hamper the normal multiplication of BHS and, thus, might be responsible for the absence of penicillin resistant BHS in nature. Thus, the possible influence of decreased susceptibility to penicillin on the growth of BHS was explored through comparison of growth rates of penicillin susceptible and variants with decreased susceptibility to penicillin.

Using the selective pressure with subinhibitory concentration of penicillin, BHS strains with decreased susceptibility to penicillin were developed. Chemical mutagens or serial passages were employed earlier to decrease susceptibility to penicillin in BHS group A strains. We chose the latter because it provided conditions more similar to those associated with naturally occurring resistance in bacteria. The observed increase in MIC values in tested strains was not continuous and the stagnation periods were probably due to adaptation to changes PBPs. The application of serial passages in the previous study resulted in increase in MIC values in BHS group A strains, but they remained susceptible to penicillin. In the present study laboratory variants of BHS group A strains with intermediate level of resistance to penicillin were developed. In all laboratory variants of other BHS serogroups, MIC values reached the level of complete resistance. Similar differences between group A strains and those of other serogroups were noted when stability of decreased susceptibility to penicillin in laboratory variants was monitored. The decreased susceptibility to penicillin in laboratory variants of BHS group A strains was lost after serial passages on the blood agar plates without penicillin. The same procedure caused only one to two concentrations decrease in MIC values in penicillin resistant variants of BHS strains serogroups B, C, and G. There is no apparent explanation for the differences observed among group A strains and those belonging to the other serogroups tested. Evaluation of susceptibility to other beta lactam antibiotics showed that induction of resistance to penicillin led to decreased susceptibility to other derivatives of penicillin and cephalosporins in laboratory variants of BHS strains. Probably subcultivation of the isolates in the presence of penicillin induced considerable multiple changes in PBPs.

Significant changes were observed in morphological and cultural characteristics of variants with decreased susceptibility to penicillin. However, all the observed alterations diminished during the subcultivation without penicillin. Reversibility of these changes indicated their phenotypic origin.

The growth rates of parental and laboratory variants of BHS strains were evaluated in three different media by two methods in order to avoid possible errors. The first method, based on counting colonies, registered the number of living bacterial cells, while the second one, based on measurement of optical density, registered both living as well as dead cells. Moreover, the typical occurrence of streptococci in chains might have been a limitation for optical density measurements. The differences observed between the growth rates of parental and laboratory variants were not significant and cultivation of penicillin susceptible and variants with decreased susceptibility to penicillin in a highly nutritious supplemented medium such as...
THB-HS, revealed that in the presence of high quantity of nutrients laboratory variants grew as well as penicillin susceptible.

In conclusion, the results of this study provided no support to the assumption that variants of BHS with decreased susceptibility to penicillin are incapable for normal growth. Further studies including large series of isolates analysed under both *in vitro* and *in vivo* conditions are needed to be done to explore the association between the decreased susceptibility to penicillin in BHS, particularly in BHS group A, and possible defects in their growth capacity and virulence.

References


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