A Low-Affinity Penicillin-Binding Protein 2x Variant Is Required for Heteroresistance in \textit{Streptococcus pneumoniae}

Hansjürg Engel, Moana Mika, Dalia Denapaite, Regine Hakenbeck, Kathrin Mühlemann, Manfred Heller, Lucy J. Hathaway, Markus Hilty

Institute for Infectious Diseases, Faculty of Medicine, University of Bern, Bern, Switzerland; Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; Department of Microbiology, University of Kaiserslautern, Kaiserslautern, Germany; Department of Infectious Diseases, University Hospital and University of Bern, Bern, Switzerland

Heteroresistance to penicillin in \textit{Streptococcus pneumoniae} is the ability of subpopulations to grow at a higher antibiotic concentration than expected from the MIC. This may render conventional resistance testing unreliable and lead to therapeutic failure. We investigated the role of the primary \(\beta\)-lactam resistance determinants, penicillin-binding protein 2b (PBP2b) and PBP2x, and the secondary resistance determinant PBP1a in heteroresistance to penicillin. Transformants containing PBP genes from the heteroresistant strain Spain\(^{23F}_{2349}\) in the nonheteroresistant strain R6 background were tested for heteroresistance by population analysis profiling (PAP). We found that \(pbp2x\), but not \(pbp2b\) or \(pbp1a\) alone, conferred heteroresistance to R6. However, a change of \(pbp2x\) expression was not observed, and therefore, expression does not correlate with an increased proportion of resistant subpopulations. In addition, the influence of the CiaRH system, mediating PBP-independent \(\beta\)-lactam resistance, was assessed by PAP on \(ciaR\) disruption mutants but revealed no heteroresistant phenotype. We also showed that the highly resistant subpopulations (HOM\(^*\)) of transformants containing low-affinity \(pbp2x\) undergo an increase in resistance upon selection on penicillin plates that partially reverts after passaging on selection-free medium. Shotgun proteomic analysis showed an upregulation of phosphate ABC transporter subunit proteins encoded by \(pstG\), \(phoU\), \(pstB\), and \(pstC\) in these highly resistant subpopulations. In conclusion, the presence of low-affinity \(pbp2x\) enables certain pneumococcal colonies to survive in the presence of \(\beta\)-lactams. Upregulation of phosphate ABC transporter genes may represent a reversible adaptation to antibiotic stress.

\textit{Streptococcus pneumoniae} is an important human pathogen causing up to 11% of child deaths per year (1). Although initially very susceptible to penicillin, resistance to the antibiotic in \textit{S. pneumoniae} has become a global concern within the last few decades. Today, a small number of resistant clones dominate the global resistance epidemiology (2, 3). The three main penicillin resistance determinants are altered penicillin-binding protein 2x (PBP2x), PBP2b, and PBP1a, which are responsible for the final cross-linking of the peptidoglycan in the bacterial cell wall (4, 5). PBP variants with low affinity for penicillin are acquired by horizontal gene transfer, followed by homologous-recombination events with commensal streptococci as donor species, giving rise to mosaic genes (4, 6–8). As detected for a clone of serotype 23F, which spread intercontinentally, the presence of low-affinity variants confers increased penicillin resistance (9).

Besides PBPs, mosaic structures in the first cell wall branching enzyme (MurM) allow pneumococci to synthesize branched cell wall muropeptides, which contribute to high penicillin resistance in some strains (10, 11). In resistant laboratory mutants, mutations in the two-component signal transduction system CiaRH (competence induction and altered cefotaxime susceptibility) that also mediate \(\beta\)-lactam resistance have been identified. In addition, CiaRH is implicated in maintenance of cell integrity, competence, and virulence (12–14). Thus, many resistance components have to be optimized for high penicillin resistance to occur. However, other resistance determinants have also occasionally been described in resistant strains (15).

Heteroresistance is thought to facilitate the development of high penicillin resistance (16). A heteroresistant bacterial strain has one or several subpopulations at a frequency of \(10^{-7}\) to \(10^{-3}\) that can grow at higher antibiotic concentrations than predicted by the MIC for the majority of cells, which are all identical genetically. Most studies focus on heteroresistance to methicillin, oxacillin, and vancomycin in staphylococci (17–23), but the phenomenon has been described for pathogens of various species, such as \textit{Acinetobacter baumannii} (24, 25), \textit{Pseudomonas aeruginosa} (26, 27), \textit{Enterococcus faecium}, and \textit{Mycobacterium tuberculosis} (28, 29), and also for fungi, such as \textit{Cryptococcus} spp. (30, 31). In pneumococci, heteroresistance to penicillin and fosfomycin has been reported and is likely to be produced via distinct mechanisms (16, 32). Understanding heterogeneity between single cells is challenging, as conventional assays of microbial populations consider averaged values of thousands or millions of cells (33). Therefore, the mechanism of heteroresistance remains unclear. A categorization into four classes according to the frequencies of subpopulations with higher resistance has been suggested based on work on methicillin-resistant staphylococci (23). For pneumococci, class II and class III heteroresistances have been observed (16). A strain with class II heteroresistance grows subpopulations with higher...
TABLE 1 Strains of *S. pneumoniae* used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST*</th>
<th>Serotype*</th>
<th>MIC of penicillin (µg/ml)</th>
<th>Frequency of cells with higher penicillin resistance</th>
<th>Heteroresistance class</th>
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<td>1.5</td>
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**pbp2x** transforms

<table>
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<td>II</td>
<td>This study</td>
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**ciaR** disruption mutants

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<th>MHST*</th>
<th>Serotype*</th>
<th>MIC of penicillin (µg/ml)</th>
<th>Frequency of cells with higher penicillin resistance</th>
<th>Heteroresistance class</th>
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<td>23F</td>
<td>0.012</td>
<td>10^{-2}-10^{-4}</td>
<td>II</td>
<td>This study</td>
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</table>

* MLST, multilocus sequence type.
* NT, nontypeable.
* MIC as determined by the Etest, which was performed three times. The values were within one doubling dilution.
* Frequency of subpopulations with higher penicillin resistance levels as determined by PAP.
* MIC for the subpopulation growing in the zone of inhibition of the Etest.

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Resistance at a frequency of 10^{-6} to 10^{-4}. Furthermore, the presence of several subpopulations with different MICs is characteristic, leading to a continuous decreased frequency of subpopulations in the population analysis profiling (PAP) curve. In contrast, in a class III heteroresistant strain, one subpopulation, represented by a plateau in the PAP curve, is predominant.

In this study, we uncover a mechanism of penicillin heteroresistance in *S. pneumoniae* by transferring *pbp* genes between heteroresistant and nonheteroresistant strains and by a shotgun proteomic approach to study the highly resistant subpopulations (HOM*).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Spain23F and Spain6B, two international reference strains of *S. pneumoniae*; the laboratory strain R6; and a selection of transformants and progeny of these strains were used in this study. All the strains used to investigate the heteroresistance phenomenon are listed in Table 1. The strains used for cloning are listed in Table S1 in the supplemental material. Bacterial conservation and growth procedures have been described previously (16) and are briefly discussed in the supplemental material.

**Antibiotic susceptibility testing.** MICs were determined by the Etest method (bioMérieux, Switzerland) according to the manufacturer’s protocol. All isolates were tested in triplicate, and the plates were incubated at 35°C.

**DNA techniques.** Pneumococcal chromosomal DNA or cell pellets were used as PCR templates (34). DNA fragments were amplified with high-fidelity iProof polymerase (Bio-Rad). The constructs were fused with either iProof or Phusion high-fidelity polymerase (Thermo Scientific) as described previously (35). The introduced genes were sequenced with either iProof or Phusion high-fidelity polymerase (Thermo Scientific) as described previously (36) to confirm correct insertion and absence of additional mutations. The DNA oligonucleotides used for PCR and sequencing are listed in Table S2 in the supplemental material.

**Transformation procedure.** Transformation of *S. pneumoniae* was performed according to published procedures (37, 38). The β-lactam concentrations used to select mosaic *pbp2x* and *pbp2x* are specified below. Streptomycin (Chemical Abstracts Service [CAS] no. 3810-74-0), kanamycin (CAS 25389-94-0), and spectomycin (CAS 22189-32-8), all from Sigma, were used at 200 µg/ml.

**Introduction of low-affinity mosaic PBP2b**. First, a 1,858-bp gene fragment containing the mosaic block was amplified from *S. pneumoniae* Spain23F using Taq polymerase (Qiagen) and primers *pbp2b_for* and *pbp2b_rev*. Then, the PCR product was cloned into pGEM-T Easy (Promega), creating plasmid pGEM-2bRes. Escherichia coli DH5α was transformed with the ligation product and selected on a 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal)/isopropyl-β-D-thiogalactopyranoside (IPTG)-LB agar plate containing 100 µg/ml ampicillin. Next, *S. pneumoniae* R6 was transformed with pGEM-2bRes, and the resistant clones were selected on citrated sheep blood agar (CSBA) plates containing 0.05 µg/ml piperacillin (CAS 59703-84-3). One trans-
formant, R6:pbp2b\_2349, which contains a complete mosaic block (codon 982 to 1472) from Spain2349 was used for further study.

Introduction of mosaic PBP1a\_2349. Transformants R6:pbp1a\_2349 and R6:pbp2b\_2349 were constructed as described previously (34). The Janus cassette was amplified from R6\_pbp2\_X1386\_pbp1\_Janus::Janus with primers Ja\_pbp1\_for and Ja\_pbp1\_rev. The presence of pbp1a\_2349 was verified in the resulting mutants by DNA sequencing. Construction of R6:pbp2\_X2349, R6:pbp2b\_X2349, and the mosaic PBP2\_X2349 transformants, and the loss-of-function CiaR derivative was done as described in the supplemental material.

PAP. PAP was performed for penicillin as described previously (16). Briefly, strains were streaked out from frozen stock on CSBA plates and incubated for 24 h in a 5% CO2 atmosphere at 37°C. Then, an overnight culture of 5 ml brain heart infusion (BHI) (BD Difco) with 5% fetal bovine serum (FBS) (Biochrom AG, Germany) was prepared and inoculated with 5 to 20 colonies. One hundred microliters of overnight culture was added to 10 ml RNA Protect (Qiagen), RNA was extracted, and expression of pstB, pstS, pbp1a, pbp2x, and pbp2b was quantified by real-time reverse transcription (RT)-PCR as described previously (39) (for the primers and probes, see Table S2 in the supplemental material). The remaining culture was pelleted and then resuspended in 200 μl PBS plus 15% glycerol and frozen at −80°C.

Detection of penicillin-binding proteins and comparison of protein expression patterns by liquid chromatography-tandem mass spectrometry (LC–MS-MS). Penicillin-binding proteins were detected as described previously (38) (A brief description is given in the supplemental material.) The LC–MS-MS method is described in the supplemental material.

RESULTS

Heteroresistance to penicillin depends on the presence of low-affinity PBP2x. First, we looked at the roles of the primary and secondary resistance determinants, PBP2b, PBP2x, and PBP1a variants with low affinity for penicillin, in heteroresistance to penicillin. Transformants carrying mosaic blocks of the pbp genes of the heteroresistant strain Spain2349 in the background of the nonheteroresistant laboratory strain R6 were characterized in PAP. Comparing the single transformants carrying pbp2b\_2349 or pbp1a\_2349, no difference in the heteroresistance phenotype from that of strain R6 was observed (Fig. 1A). However, R6:pbp2x\_2349 showed class II heteroresistance, characterized by growth of subpopulations with higher resistance at a frequency of 10−4 to 10−3 (Table 1 and Fig. 1A). The double transformants carrying pbp2b\_2349 and pbp1a\_2349 in the R6 background showed no heteroresistance, meaning no subpopulations growing with higher resistance than the MIC (Fig. 1B). However, the combination of pbp2b\_2349 and pbp2x\_2349 led to class II heteroresistance, characterized by the presence of several subpopulations at a frequency of 10−4 to 10−1 (Table 1). The transformant with pbp2x\_2349 and pbp1a\_2349 showed more highly resistant subpopulations at a frequency 10−3 to 10−4 in a class III heteroresistance pattern characterized by the growth (dominance) of one subpopulation. Uniting the three resistance genes in a triple transformant led to a phenotype close to that of wild-type (WT) Spain2349 in the background of the nonheteroresistant strain R6 compared to the R6 wild type (A), double transformants with two pbp\_2349 compared to R6 (B), and a triple transformant containing all three pbp genes in the R6 background compared to Spain2349 and the R6 wild type (C). The concentration of penicillin G used to select subpopulations with higher penicillin resistance levels is shown against the frequency of bacteria able to grow at that concentration. Representatives of three independent experiments are shown. The replicates are shown in Fig. S1 and S2 in the supplemental material.
Performing PAP, we found that combining $pbp2b_{2349}$ and $pbp2x_{6B}$ in the R6 background (Fig. 2A; see Fig. S4 in the supplemental material) leads to class II heteroresistance, as observed for the combination $pbp2b_{2349}$ plus $pbp2x_{2349}$ (Fig. 1B). Identical findings were also obtained when combining $pbp1a_{2349}$ and $pbp2x_{6B}$, as this led to class III heteroresistance, as seen with $pbp2b_{2349}$ plus $pbp2x_{2349}$ (Fig. 2B; see Fig. S4 in the supplemental material). Introduction of $pbp2x_{6B}$ into R6, therefore, led to a heteroresistance pattern identical to that with $pbp2x_{2349}$. Compared to Spain$^{6B-2}$ and Spain$^{23F}$, nonheteroresistant strain R6 has a unique transpeptidase domain, while its PASTA domain is nearly equal to that of Spain$^{6B-2}$ (with the exception of amino acid 693 [see Fig. S3 in the supplemental material]). This therefore shows that the transpeptidase domain of PBP2x, but not the two C-terminal PASTA domains, is involved in the heteroresistance phenotype.

**ciaR disruption has no influence on the heteroresistance phenotype.** As the CiaRH system can mediate PBP-independent β-lactam resistance, we investigated whether heteroresistance was lost upon silencing the CiaRH system by disruption of the response regulator gene $ciaR$. It was found that $ciaR$ disruption did not affect MICs (Table 1), although in PAP, R6Δ$ciaR$ displayed a slightly higher susceptibility to penicillin than the R6 WT. However, R6$pbp2x_{2349}$Δ$ciaR$ retained heteroresistance to penicillin compared to R6$pbp2x_{2349}$ (Fig. 2C; see Fig. S4 in the supplemental material).

**PAP of HOM* progeny strains shows a shift toward higher resistance, which reverts partially after passaging on selection-free medium.** From PAP, HOM* progeny strains were obtained. HOM* progeny strains were grown from a single colony picked from a PAP plate with the highest or second-highest penicillin concentration showing growth. The original and progeny of double transformants harboring $pbp2b$ and $pbp2x$ of heteroresistant strain Spain$^{23F}$ in the R6 background (A) and $pbp2x_{2349}$ (B) are shown. Additional HOM* lineages are shown in Fig. S5 in the supplemental material.

![FIG 2](image1)

**FIG 2** Influence of the $pbp2x$ gene sequence, genetic background, and CiaRH system on heteroresistance. (A and B) PAP for penicillin of transformants harboring $pbp2b$ of heteroresistant strain Spain$^{23F}$ plus $pbp2x$ of nonheteroresistant strain Spain$^{6B-2}$ (A) and $pbp1a_{2349}$ plus $pbp2x_{6B}$ (B) in the background of nonheteroresistant strain R6. (C) Mutants with the CiaRH system silenced through disruption of $ciaR$ in mutants of R6$pbp2x_{2349}$ and R6 compared to the original strains. The replicates are shown in Fig. S4 in the supplemental material.

![FIG 3](image2)

**FIG 3** PAP for HOM* strains of double transformants with heteroresistance to penicillin. HOM*1, HOM*2, and HOM*3 are derivatives of the respective strains obtained by selection of single colonies during successive PAP experiments. HOM*3p is progeny of HOM*3 that has been passaged 15 times on selection-free medium. Single colonies were picked from plates with the highest or second-highest penicillin concentration showing bacterial growth. The original and progeny of double transformants harboring $pbp2b$ and $pbp2x$ of heteroresistant strain Spain$^{23F}$ in the R6 background (A) and $pbp2x_{2349}$ (B) are shown. Additional HOM* lineages are shown in Fig. S5 in the supplemental material.
μg/ml penicillin for HOM*1 to 0.38 μg/ml penicillin for HOM*3 and reverted to 0.064 μg/ml for HOM*3p. For HOM*2, HOM*3, and HOM*3p, subpopulations grew at concentrations up to 2 to 7 times the MIC determined for the majority of cells within the inhibition zone of the Etest (Table 1). Again, replicates of PAP of R6::pbp2b2x2349 and its HOM* were highly reproducible (see Fig. S5 in the supplemental material). Therefore, both R6::pbp2b2x2349 (Fig. 3A) and R6::pbp2x1a2349 (Fig. 3B) shift toward higher resistance despite the heteroresistance class difference between the two original transformants (Fig. 1B). Also, MIC values measured for HOM*3p switch back to initial levels (Table 1).

**Altered growth for HOM* progeny strains.** For PAP, CFU are counted after 48 h of incubation at 37°C to account for potential reduced growth of the subpopulation strains with higher resistance. However, to characterize more precisely differences in growth phenotypes for the double-transformant HOM*3 and HOM*3p progeny, growth curves of these strains were obtained. The original strains both grew to a maximum OD450 of 0.3 (see Fig. S6 in the supplemental material). However, the R6::pbp2b2x2349 HOM*3 strain showed a tendency to grow to a higher OD than the original transformant (see Fig. S6A in the supplemental material), whereas for R6::pbp2x1a2349, the opposite was observed. The R6::pbp2b2x2349 HOM*3p strain grew in a similar way to the original transformant. Finally, the HOM*3p progeny R6::pbp2x1a2349 exhibited clearly impaired growth (see Fig. S6B in the supplemental material).

**Altered protein expression levels in highly resistant subpopulation progeny strains.** In order to identify components that might be responsible for the highly resistant subpopulations, the protein profiles of the R6::pbp2b2x2349 original transformant and its HOM*3 progeny were investigated by a shotgun LC–MS-MS analysis. All of the quantifiable proteins (899 in total) had expression differences smaller than 1.5 (0.5 in log2 values, as displayed in Fig. 4A). A small fraction of 15 proteins had a statistically significant expression difference between the two samples ($P < 0.05$; Students t test) (see Table S3 in the supplemental material). Within these, four, which are all subunit proteins encoded by the same phosphate ABC transporter operon, had a HOM/WT ratio of 0.25 (the genes were annotated as pstS, phoU, pstB, and pstC) (Fig. 4A). No significant differences were observed for the penicillin-binding proteins in this shotgun proteomic analysis. The expression of two of the four genes (pstS and pstB) was subsequently
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quantified by real-time RT-PCR for strain R6::pbp2b2x2349 and its HOM*1 and HOM*3 progeny (Fig. 4B). While no difference in gene expression was measured for HOM*1 progeny, HOM*3 progeny expressed pstS and pstB to about 100-fold-higher levels than the unselected population. We were able to confirm the increased expression for pstS and pstB for the WT (low expression) and HOM*3 (high expression) in two additional lineages (see Fig. S7 in the supplemental material). As for HOM*1, the findings are in contrast to those of the original experiment (Fig. 4B), as we observed increased expression. However, heterogeneity for HOM*1 may be interpreted as a transition state between the WT and HOM*3. The RT-PCR results, therefore, matched the LC–MS–MS results, showing significant upregulation of pstS and pstB in HOM*3. No difference in pstS and pstB expression was detected in the R6 WT compared to the single and double mutants (data not shown).

No differences in the expression of penicillin-binding proteins. Although LC–MS–MS did not indicate any differences in the expression of the penicillin-binding proteins, their expression was quantified and compared between the double transformants and their HOM*3 progeny strains using a different method. Production of PBPs was determined by staining with BocillinFL, a fluorescence-labeled β-lactam, and separation by SDS-PAGE. However, no increase in PBP was detected in HOM*3 progeny compared to the original double transformants (Fig. 4C). This was also true when measuring pbp gene expression by real-time RT-PCR (Fig. 4D).

DISCUSSION

Heteroresistance describes the presence of one or several subpopulations of bacterial cells within a clonal strain that can grow at antibiotic levels higher than those determined for the majority of cells. The phenomenon has been described for pneumococci without shedding light on the molecular mechanism (16). In this study, we aimed to identify the relevant mechanisms that produce heteroresistance to penicillin.

Our data suggest that a low-affinity variant of PBP2x is required for a heteroresistant phenotype, which therefore assumes a key role in heteroresistance to penicillin. This finding is similar to previous work in staphylococci, where heteroresistance to methicillin was observed upon insertion of mecA-encoded PBP2a (19, 22). Interestingly we found the combination of low-affinity PBPs to determine the heteroresistance class of a strain and therefore the frequency of heteroresistant subpopulations, as established in Staphylococcus aureus (23). As observed previously, the expressed heteroresistance class is a stable phenotypic trait (16, 23). PBP2b and PBP2x are monofunctional enzymes catalyzing transpeptidation only, whereas PBP1a exhibits transpeptidation and transglycosylation activities. It has been suggested that at penicillin concentrations close to the MIC, the transglycosylation activity of PBP1a, which is not targeted by β-lactams, confers a critical degree of cell wall integrity for growth, as peptidoglycan is incompletely cross-linked because PBP transpeptidase activity is hampered by penicillin (40, 41). Hence, in the R6::pbp2b2x2349 transformant, only the transpeptidase activity of the susceptible PBP1a is inhibited by penicillin. The low-affinity PBP2x is not inhibited by penicillin. This might to some degree replace this function (42). In R6:: pbp2x1a2349, however, the essential PBP2b is targeted by penicillin (43). The subpopulations with higher resistance, therefore, arise at lower frequency, but when they occur, they can grow to higher resistance levels. This is in agreement with the observation that modified PBP1a is required for high penicillin resistance (44). We report low-affinity PBP2x to be an essential tool in the production of the penicillin-heteroresistant phenotype.

It has been hypothesized that auxiliary resistance genes in concert with low-affinity PBP variants produce a heteroresistant phenotype (16). Therefore, we also disrupted ciar to test the influence of a silenced CiaRH system, which mediates PBP-independent β-lactam resistance (12), on the heteroresistant phenotype. We found heteroresistance to be conserved and conclude that the CiaRH system has a negligible effect on the phenomenon. A previous study reported that total PBP amounts found within bacteria do not differ between resistant and susceptible pneumococci, nor does the amount increase when a subinhibitory concentration of penicillin is present in the growth medium (45). We confirmed this finding, as we could not detect increased amounts of PBP or overexpressed pbp genes.

However, proteomic analysis of the R6::pbp2b2x2349 original transformant and its HOM*3 progeny revealed significant overexpression of some phosphate ABC transporter subunit proteins. The functions of most of these components are unknown. However, recently, increased expression of pstS has also been identified by proteomic analysis for a clinical isolate of serotype 23F (46). This is intriguing, as the common finding between our group and that of Soualhine et al. clearly shows the importance of pstS for both penicillin resistance and heteroresistance within S. pneumoniae. Soualhine et al., furthermore, described an excellent correlation between resistance and increased expression of pstS by RT-PCR (46). In contrast, we did not find any expression differences between the R6 WT and the single and double mutants. However, if and how the presence of different penicillin-binding proteins affects pstS expression needs to be further investigated.

In conclusion, we show the importance of classical resistance mechanisms, represented by a low-affinity variant of PBP2x, in the phenomenon of heteroresistance to penicillin. Furthermore, we detected increased expression of phosphate ABC transporter genes in the HOM* strains, representing a reversible adjustment to antibiotic stress. Improved understanding of the mechanism of heteroresistance may lead to improved diagnostics and to an adjustment of antibiotic treatment.

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