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Vancomycin-Tolerant *Streptococcus pneumoniae* in Korea

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A nationwide surveillance study was undertaken to monitor antimicrobial resistance among clinical isolates of *Streptococcus pneumoniae* in Korea, with a special focus on vancomycin tolerance. For the 6-month period from March to August 2002, clinical isolates of *S. pneumoniae* were collected from 11 university hospitals and 1 reference laboratory. One-hundred eighty-eight isolates were measured for lysis rates after exposure to vancomycin for 4 h. Two vancomycin-tolerant *S. pneumoniae* (VTSP) strains, S3 and H8, were isolated from sputum cultures of two patients, who had stayed in intensive-care units of different hospitals with long-term antibiotic therapy and were not treated for pneumococcal pneumonia. The penicillin, cefotaxime, and vancomycin MICs for S3 were 8 μg/ml, >16 μg/ml, and 0.5 μg/ml, and those for H8 were 2 μg/ml, 2 μg/ml, and 0.5 μg/ml, respectively. While S3 belonged to serotype 23F and was autolysin defective, H8 belonged to serotype 13F and had intact autolysin. These strains were not clonally related as determined by pulsed-field gel electrophoresis of chromosomal DNA. In agreement with previous reports, both isolates showed pairing of TIGR4 *vex2* with R6 *pep27* and had two identical amino acid substitutions, Q441K in *vncS* and N25D in *vex2*. These findings indicate that two VTSP strains have emerged independently in Korea, suggesting a prevalence rate of 1.1%. The emergence of VTSP would be a serious threat in Korea, where there are significant rates of penicillin resistance in *S. pneumoniae*. Monitoring of the prevalence of VTSP and further investigation of the clinical relevance of VTSP are warranted.

Streptococcus pneumoniae is the most common cause of bacterial meningitis worldwide (23, 28). Pneumococcal meningitis has a serious mortality rate, ranging from 19% to 26% (22, 23, 32). Over the past decade, the incidence of penicillin resistance in *S. pneumoniae* (PRSP) has increased dramatically, and vancomycin in combination with an extended-spectrum cephalosporin has become the treatment of choice for empirical therapy of patients with suspected or proven pneumococcal meningitis in areas where infections caused by PRSP have been documented (28). In Korea, the frequency of clinical isolates with resistance to penicillin has risen steadily, from 25% in 1990 to 71.5% in 2000 (9, 11). Moreover, 34% of clinical isolates from 1989 to 1995 (24) and 47.7% of blood isolates from 1996 to 2000 were resistant to three or more classes of antibiotics (10).

Although no vancomycin-resistant strains have been isolated to date, single therapy with vancomycin was associated with clinical failure in 4 of 11 cases of meningitis due to relatively penicillin-resistant pneumococcal strains (31). Vancomycin-tolerant *S. pneumoniae* (VTSP) has been described previously and was subsequently linked to a case of recrudescent meningitis (13). Vancomycin tolerance has been proven to play a role in therapeutic failure in an experimental rabbit model of pneumococcal meningitis (17), and patients with meningitis caused by VTSP have been shown to have a poorer survival rate than patients with meningitis caused by nontolerant strains (21). Because tolerance is a precursor to the development of resistance, these findings have important implications for the use of vancomycin for pneumococcal meningitis. The emergence of VTSP would be a serious threat in countries with significant rates of PRSP (29).

We conducted a nationwide surveillance study of antimicrobial resistance among clinical isolates of *S. pneumoniae* to monitor the emergence of vancomycin-tolerant strains.

MATERIALS AND METHODS

Bacterial strains. For the 6-month period from March to August 2002, clinical isolates of *S. pneumoniae* were collected from 11 university hospitals located in four metropolitan cities and four major cities of three provinces and from one nationwide reference laboratory in Korea. Among these, four hospitals were located, respectively, in the four boroughs of metropolitan Seoul. As many as 20 strains were collected from each institute, with a rule of one strain per patient. The strains were stored in brain heart infusion broth with 20% glycerol at −70°C. R6 and Lyt4-4, a LytA-defective laboratory mutant of R6, were used as negative and positive controls for vancomycin tolerance, respectively (26). All strains were subcultured on blood agar plates at 37°C under 5% CO_2.

Antimicrobial susceptibility. All isolates were tested for susceptibility to penicillin (Sigma-Aldrich, St. Louis, Mo.), cefotaxime (Sigma-Aldrich), meropenem (AstraZeneca, Cheshire, United Kingdom), and vancomycin (Sigma-Aldrich). MICs were determined by the broth microdilution method using cation-adjusted Mueller-Hinton broth (BBL) supplemented with 2.5% lysed horse blood (16). Interpretive criteria for susceptibility were those indicated in Clinical and Lab-

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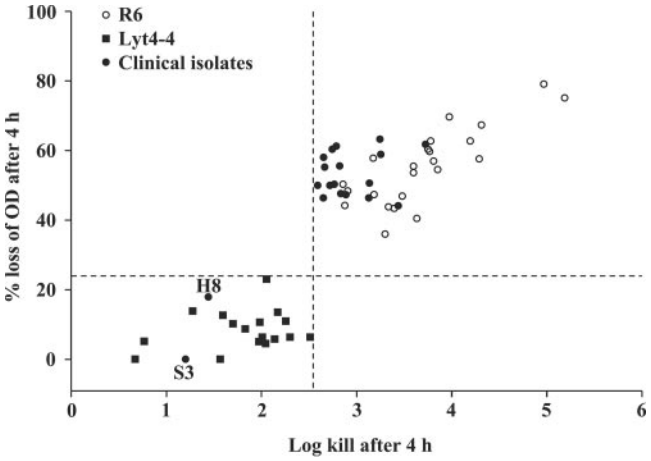

FIG. 1. Mean percent loss of OD and log kill after addition of vancomycin (at a concentration equivalent to 10 times the MIC) for 20 candidate clinical isolates of pneumococci. The values for R6 and Lyt4-4 are also shown. Isolates S3 and H8 showed rates of killing and lysis similar to those of the autolysin-defective mutant Lyt4-4. Dashed lines indicate the limits of tolerance.

oratory Standards Institute (formerly NCCLS) document M100-S16 (3). To confirm vancomycin tolerance, the minimum bactericidal concentration (MBC) of vancomycin was measured using the same media and incubation conditions as those for the MIC tests (15).

Vancomycin-induced lysis rates and definition of tolerance. Vancomycin-induced lysis rates were measured as described previously (6). Briefly, two or three bacterial colonies from the culture of each isolate were inoculated into 2 ml of semisynthetic casein hydrolysate medium (12) supplemented with 0.1% yeast extract (Difco) (c + y medium) and incubated overnight at 37°C under 5% CO_2. When the optical density (OD) of each culture at 620 nm reached 0.20 to 0.30 (5.6×10^7 to 2.7×10^8 CFU/ml), vancomycin was added at a concentration equivalent to 10 times the MIC. Loss of OD was measured hourly for 4 h and then at 20 h after the addition of vancomycin, and those isolates showing a <50% loss of OD were selected as the candidates for measurement of log kill. Cultures of candidate isolates were serially diluted in c + y medium before and 4 h after the addition of vancomycin and were then plated onto blood agar plates. Viable cells were counted after overnight incubation at 37°C under 5% CO_2, and log kill counts were determined as $\log_{10}$ decreases in viable cell counts. Log kill counts were performed in duplicate for each candidate. The lysis patterns of R6 and Lyt4-4 were determined from 20 separate experiments in order to establish the tolerance limits. After 4 h of exposure to vancomycin, the mean log kill was 4.0 (standard deviation [SD], ±0.5) and the mean loss of OD was 56.9% (SD, ±10.4%) for R6, whereas the mean log kill was 1.9 (SD, ±0.3) and the mean loss of OD was 9.1% (SD, ±7.1%) for Lyt4-4. The limit that defined vancomycin tolerance was set at 2 SDs above the means for Lyt4-4 (log kill, ≤2.5; OD loss, ≤23.3%).

Sequencing and data analysis. VTSP isolates and R6 were subjected to sequencing of *vncS*, *vex2*, and *pep27*. The primers used for PCR and sequencing of *vex2*, *pep27*, and *vncS* have been described by Rodriguez et al. (21). Sequencing was performed using the ABI PRISM dye terminator cycle sequencing ready reaction kit and AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems) on a model 373 automated DNA sequencer (Perkin-Elmer, Foster City, CA). The deduced amino acid sequences were aligned with those of reference isolates TIGR4 (GenBank accession no. NC 003028) and R6 and with those of vancomycin-tolerant strains I95, A378, and A43, published elsewhere (6).

Autolysin activity. For vancomycin-tolerant isolates, active autolysin was detected by visible clearing of the suspensions of their cultures in 2% sodium deoxycholate and by the abilities of their cellular extracts to reconstitute the penicillin-induced lysis of the autolysin-defective isolate Lyt4-4 (27).

Serotyping and PFGE. Vancomycin-tolerant isolates were serotyped by the capsular quellung method with commercial antisera (Statens Seruminstitut, Copenhagen, Denmark) as recommended by the manufacturer. The relatedness of vancomycin-tolerant isolates was determined by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA restricted with SmaI and ApaI (7).

RESULTS

Antimicrobial susceptibility. A total of 188 isolates were collected: 121 from lower respiratory tract specimens, 26 from blood cultures, 12 from middle ear aspirates, 2 from cerebrospinal fluid (CSF), and 27 from other sites. Forty-three (22.9%) isolates were from patients ≤15 years old. Among all isolates, 29.3%, 62.8%, 42.6%, and 100% were susceptible to penicillin, cefotaxime, meropenem, and vancomycin, respectively. The penicillin MICs at which 50% and 90% of the isolates were inhibited were 2 μg/ml and 4 μg/ml, respectively. Penicillin-susceptible isolates were susceptible to all four drugs. But of the 100 penicillin-resistant isolates, 70 (70%), including 2 CSF isolates, were susceptible to neither cefotaxime nor meropenem. All of the isolates had vancomycin MICs ranging from 0.12 μg/ml to 1 μg/ml.

Vancomycin tolerance. The mean loss of OD of 188 isolates at 4 h was 56.6% (SD, ±12.3%). Twenty isolates showing ≤50% losses of OD were counted for viable cells to confirm the vancomycin-induced lysis rate. Most of the 20 clinical isolates showed substantial losses of OD, and viable cell counts similar to those of R6. Only two isolates, S3 and H8, were consistent with the definition of vancomycin tolerance: the losses of OD for S3 and H8 were 0.3% and 19.2%, respectively, and the mean log kills were 1.3 and 1.5, respectively (Fig. 1). The MBC-to-MIC ratios of vancomycin were ≥64 for both (Fig. 2).

Characterization of vancomycin-tolerant strains. S3 was isolated from a sputum culture of a 91-year-old man admitted to the medical intensive-care unit of a tertiary-care hospital in Seoul, and H8 was isolated from a sputum culture of a 78-year-old man admitted to the medical intensive-care unit of a secondary-care hospital in Seoul. In both cases, *S. pneumoniae* was not the target of therapy at the time. Both patients had been repeatedly treated with various antibiotics, including cefotaxime and vancomycin, for prolonged periods prior to the isolation of *S. pneumoniae*. The MICs of penicillin, cefotaxime, meropenem, and vancomycin for S3 were 8 μg/ml, >16 μg/ml, 1 μg/ml, and 0.5 μg/ml, respectively, whereas the corresponding MICs for H8 were 2 μg/ml, 2 μg/ml, 0.5 μg/ml, and 0.5 μg/ml, respectively. The serotypes of S3 and H8 were 23F and 13F, respectively. PFGE analysis showed that they were not clonally related.

Upon exposure to deoxycholate, H8 underwent rapid lysis, like R6, whereas S3 did not, like Lyt4-4. Moreover, lysates of H8, like those of R6, reconstituted the lysis activity of the autolysin-deficient strain Lyt4-4, whereas lysates of S3 did not, indicating that S3 was autolysin defective (Fig. 3).

The sequence of R6 was consistent with the published sequence in GenBank (accession no. AE008431). Compared to the sequences of reference strains TIGR4 and R6, the sequences of S3 and H8 have identical single-amino-acid substitutions—a glutamine-to-lysine substitution at position 441 of the *vncS* gene and an asparagine-to-aspartic acid substitution at position 25 of the *vex2* gene—that differentiate them from the published sequences of vancomycin-tolerant strains I95, A378, and A43 (6). While the *vex2* alleles of S3 and H8 showed the TIGR4 type, characterized by arginine at position 102 and asparagine at position 111, their *pep27* alleles showed the R6 type, harboring a glycine-to-aspartic acid substitution at posi-

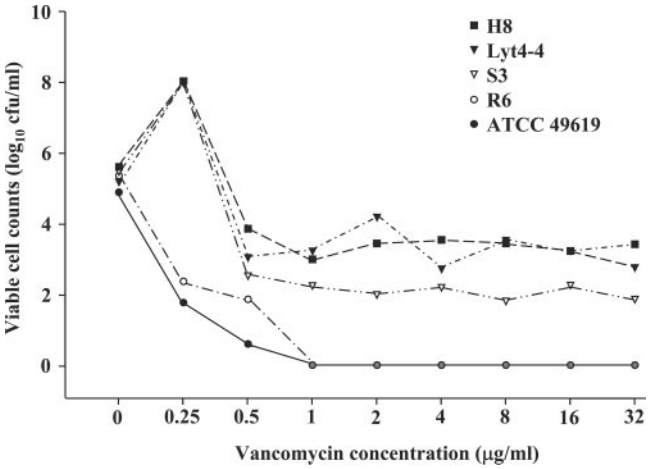

FIG. 2. MBCs of vancomycin for S3, H8, and control strains ATCC 49619, R6, and Lyt4-4. Gray circles, no growth detected on 1-ml cultures.

tion 12 and an alanine-to-tyrosine substitution at position 16 (Fig. 4).

DISCUSSION

Of our 188 clinical isolates, 2 (1.1%) were tolerant vancomycin. Because Korea is one of the countries that suffer from the highest incidences of PRSP infection (25), this prevalence of VTSP could pose serious concerns. However, this prevalence may have been overestimated, because pneumococcal isolates were mostly collected from large university hospitals, and about one third of the isolates came from tertiary-care hospitals in metropolitan Seoul. By 2003, six clinical VTSP isolates had been reported sporadically (2, 6, 8, 13). Surveillance studies from 2001 to 2005 have reported VTSP prevalences ranging from 0% to 2.6% in Sweden, Spain, Hong Kong, France, and the United States (1, 2, 4–6, 19). In 2004, Rodriguez et al. (21) reported VTSP prevalences of 8.1% among the 457 nasopharyngeal isolates collected from healthy newborns between 1999 and 2002, and 10.6% and 3.3% for 113 blood and CSF isolates and 215 nasopharyngeal isolates of healthy infants, respectively, retrieved from the archival collection of 1998. However, they modified the criteria for defining tolerance from <2 SDs above the mean log kill of Lyt4-4 (6) to <2 SDs below that of R6 (21). Differences in VTSP prevalence may depend on the methods used to define vancomycin tolerance, as well as on the patient population and its geographic distribution. To estimate the prevalence of VTSP reliably, the method and criteria used to define vancomycin tolerance must be standardized.

To our knowledge, S3 was the first clinical isolate of autolysin-defective VTSP. Although the exact mechanism underlying vancomycin tolerance is still unclear, it is apparently related to a defect in autolysis (6, 17). The lack of autolysin alone can explain vancomycin tolerance, as exemplified by the original *lytA* defective mutant (27). Even though two VTSP isolates emerged from two patients independently and were not clonally related, they shared several genetic characteristics. In this study, we detected novel single-amino-acid substitutions, Q441K of *vncS* and N25D of *vex2*, in both S3 and H8. VncS-VncR, Vex, and Pep27 are involved in the signal transduction cascades to activate autolysin (LytA). VncS is a histidine kinase that acts as the sensor of a two-component regulatory system sensing the accumulation of Pep27 and subsequently leading to activation of LytA. Vex is the transporter protein inducing the secretion of Pep27 to the extracellular compartment (14, 17, 18). The V440A substitution in *vncS* has been proposed to cause tolerance in early VTSP isolates (6). A functional defect in any part of these signal transduction cascades hypothetically results in tolerance (18). However, loss of VncS function alone has not been proven to cause vancomycin tolerance, and the frequencies of V440A were found to be similar in tolerant and nontolerant isolates (20, 21). We did not investigate the effect of the novel amino acid substitutions found in this study. Both isolates S3 and H8 showed a combination of a TIGR4 *vex2* allele and an R6 *pep27* allele. This finding is consistent with the report of Rodriguez et al. that vancomycin-tolerant isolates show a significant tendency to have this combination (21). As a summary, the vancomycin tolerance found in this study may possibly be attributed to any of three kinds of mechanisms: a defect in autolysis in one isolate, a functional defect in the VncS-VncR system, and the combination of TIGR4 *vex2* and R6 *pep27* alleles.

Two vancomycin-tolerant strains were highly resistant to penicillin and cefotaxime. Among 188 clinical isolates, the prevalences of penicillin-intermediate and -resistant *S. pneumoniae* were 17.6% and 53.2%, respectively, findings similar to those of previous reports (9, 10, 25). Published cases of VTSP were all penicillin intermediate or penicillin resistant (2, 6, 8, 13). These findings are consistent with the presumption that selective pressure by antibiotics accelerates the development of tolerance and resistance (21). Due to the very high rate of β-lactam resistance, a high risk of emergence of VTSP may be anticipated. VTSP emergence could seriously limit the therapeutic options for *S. pneumoniae* infection in Korea.

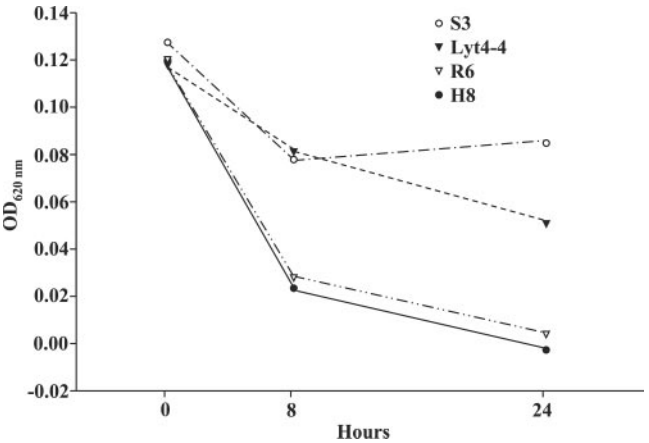

FIG. 3. Functional assay of autolytic activity of vancomycin-tolerant strains by reconstitution of penicillin-induced lysis of Lyt4-4. Crude cellular extracts of S3, H8, R6, or Lyt4-4 were added to cultures of Lyt4-4 together with 0.1 μg/ml penicillin, and then the OD at 620 nm was measured at 0 h, 8 h, and 24 h.

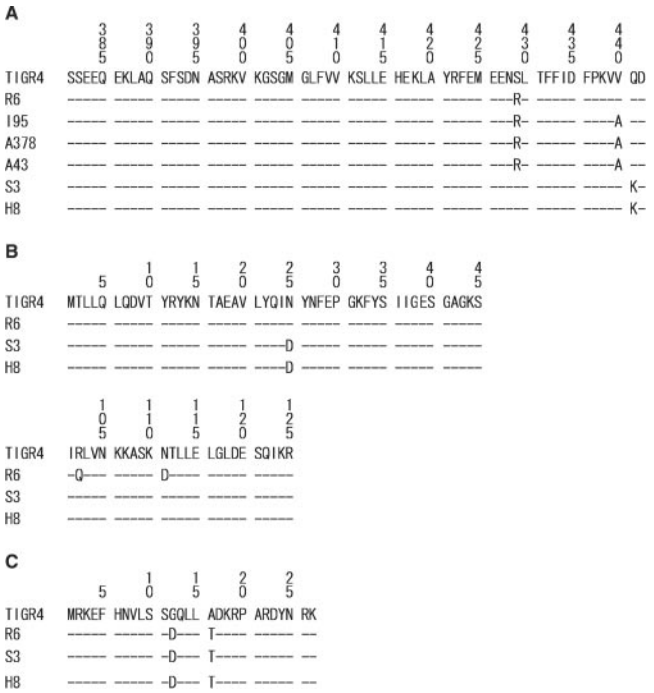

FIG. 4. Amino acid sequences of the C-terminal regions of *vncS* (A), *vex2* (B), and *pep27* (C). The deduced sequences from *S. pneumoniae* R6, S3, and H8 were aligned with those of *S. pneumoniae* TIGR4 (GenBank accession no. NC 003028). Dashes represent amino acids identical to those of TIGR4. The sequences of vancomycin-tolerant isolates I95, A378, and A43 (6) were aligned for comparison of the C-terminal regions of *vncS* sequences.

Because the patients from whom S3 and H8 was isolated were not being treated for pneumococcal pneumonia, the clinical impact of VTSP could not be assessed in this study. Except for a case of pneumococcal meningitis, the clinical relevance of vancomycin tolerance has not been investigated yet (12, 21). In the previous report investigating lysis-defective strains, a defect in penicillin-induced lysis was shown to affect the course of meningitis adversely, despite appropriate β-lactam treatment, but not that of bacteremia (30). To establish the clinical relevance of VTSP, a much larger study involving more cases of VTSP infection would be required.

In conclusion, VTSP, with a prevalence of 1.1% in this study, has emerged from PRSP in Korea. Given the high incidence of penicillin resistance, the emergence of VTSP is more likely and furthermore would have a greater impact on therapeutic options for pneumococcal meningitis in Korea. Further monitoring of the prevalence, and further investigation of the clinical relevance, of VTSP is warranted.

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