Cross-Sectional Study of Nasopharyngeal Carriage of Streptococcus pneumoniae in Human Immunodeficiency Virus-Infected Adults in the Conjugate Vaccine Era

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Human immunodeficiency virus (HIV)-infected patients have an increased rate of pneumococcal infections. Within the HIV-infected population, patients with low CD4+ cell counts have a higher rate of pneumococcal infection. The purpose of our study was to determine pneumococcal carriage and to examine the serotypes carried by HIV-infected patients after the introduction of the conjugate vaccine. Nasopharyngeal swabs were obtained from patients during routine clinic visits. Samples were cultured on blood agar plates with gentamicin and screened for alpha-hemolysis, optochin sensitivity, and bile solubility. Capsular serotypes were determined by multiplex PCR, multilocus assay, or latex agglutination. Antibiotic susceptibility was determined by the Etest method. Multilocus sequence typing was also performed. Of the 175 patients enrolled, 120 patients had absolute CD4+ cell counts above 200/mm³ and 55 had counts below 200/mm³. A total of six (3.4%) patients carried pneumococci. All but one of these patients had received the 23-valent pneumococcal vaccine within the previous 5 years. Five of the isolates were serotypes that are not included in the 7-valent conjugate vaccine. Immunization with the pneumococcal polysaccharide vaccine does not prevent colonization in HIV-infected patients; however, the observation of carriage of serotypes not included in the conjugate vaccine may be due to herd immunity and serotype replacement effects in the general population.

Streptococcus pneumoniae, also referred to as pneumococcus, is a major worldwide cause of morbidity and mortality (36). Invasive pneumococcal disease (IPD) may manifest as pneumonia, bacteremia, or meningitis, all of which are potentially fatal infections. Pneumococcal infections are more prevalent in otherwise healthy populations of young children (<5 years old) and the elderly (≥65 years old) (36). However, there is an increased risk of invasive disease in individuals who are immunocompromised (21). Human immunodeficiency virus (HIV)-infected individuals are one such group in which rates of IPD are increased as much as 100-fold compared to the general population (17, 18). Within the HIV-infected population, absolute CD4+ cell counts may be a factor in IPD risk: those with lower CD4+ cell counts have higher rates of disease than those with higher CD4+ cell counts (2, 7).

One reason for the increased rate of invasive disease is, obviously, immunosuppression in these individuals. Another contributing factor may be an increased rate of carriage among this population. It is widely believed that carriage must be established before pneumococcal disease can develop (3). Rodriguez-Barradas study (32) occurred before the implementation of the pneumococcal conjugate vaccine. The purpose of our study, therefore, was to examine carriage in an HIV population and also determine the epidemiology of serotype changes since the licensure of the conjugate vaccine.

Two features of that study is that it focused solely on HIV-infected men, even though now about half of all HIV-infected individuals worldwide are women (35). Also of note is that the Rodriguez-Barradas study (32) occurred before the implementation of the pneumococcal conjugate vaccine. The purpose of our study, therefore, was to examine carriage in an HIV population and also determine the epidemiology of serotype changes since the licensure of the conjugate vaccine.

One sample per patient was collected from the nasopharynx by using a calcium alginate swab (Fisher Scientific, Suwanee, GA; Spectrum Laboratories, Dallas, TX) and was plated immediately onto tryptic soy agar plates containing 5% sheep blood and 2.5 μg/ml gentamicin (Becton-Dickinson, Sparks, MD). Plates were incubated at 37°C in 5% CO₂ for 48 h. In most cases, only one colony was chosen per plate; more were selected if colonies differed in morphology. S. pneumoniae isolates were identified by alpha-hemolysis, optochin sensitivity, and bile solubility. Pneumococcal isolates were grown in Todd-Hewitt broth plus 0.5% yeast extract and stored in 20% glycerol at −80°C.

Capsule typing. DNA was isolated using a Qiagen DNAeasy kit (Valencia, CA) according to the provided protocol. Initial capsule typing was performed using the multiplex PCR method, followed by the multiplex bead assay as described in the multiplex PCR method, followed by the multiplex bead assay as described.
Previously, with purified DNA as the template in the PCR instead of cell lysates (26, 27, 39). Strains that could not be typed by either method were sent to the Centers for Disease Control and Prevention (CDC) for further typing by latex agglutination and the Quellung reaction.

**Antibiotic susceptibility.** Isolates were tested for antibiotic susceptibility to penicillin, azithromycin, TMP-SMX, and moxifloxacin using the Etest (AB Biodisk North America, Piscataway, NJ) method according to manufacturer's instructions, using 5% horse blood (Quad Five, Ryegate, MT) for all antibiotic testing. MICs were determined, and susceptibility was assigned based on the 2008 Clinical and Laboratory Standards Institute guidelines (6).

**MLST.** Pneumococci were subjected to analysis by multilocus sequence typing (MLST) according to the instructions on the MLST website (http://www.mlst.net/). Briefly, PCR was performed to amplify seven pneumococcal housekeeping genes (aroE, ddl, gdh, gki, recP, spi, and xpt). These genes were then sequenced (SeqWright, Inc., Houston, TX) and aligned using the Basic Local Alignment Search Tool from NCBI. The resulting allelic profiles were concatenated to confirm pneumococcal identity (12) and analyzed to determine sequence type.

### RESULTS

**Patient population.** In this study, 175 HIV-infected patients were enrolled, ranging in ages from 18 to 66 years old (Table 1). There were almost three times as many males in our HIV-infected population than females. In our population, 82% were African-American; the majority of the other patients were white, but there were also two Hispanic patients and one Native American. One hundred thirty-six patients had received PPV23 within the past 5 years, and eight had received the vaccine at least 5 years prior; only 24 patients had not received it (there were seven patients with incomplete records who were unsure of their vaccination history). Seventy-seven percent of all patients were being treated with antiretroviral therapy, while the remainder had either never been placed on HAART (13.7%) or had been nonadherent with treatment. One-fifth of patients with CD4+ cell counts of <200/mm³ were not on any prophylactic antibiotics. This was because of medication allergies or because the patients did not consistently present for clinic appointments. Slightly more than half of the patients reported a history of smoking (including three who quit within the last 6 months).

**Capsule typing.** We provisionally identified 22 isolates as *S. pneumoniae* by optochin susceptibility and bile solubility. These isolates were then typed by multiplex PCR (Fig. 1). We were able to classify five isolates by this method as 19F, 19A, 11A, and 35B (Table 2). The nontypeable isolates were positive for the *cpsA* gene (the capsule gene conserved in all serotypes used as an internal control). All isolates were then examined by the multibead assay. The isolates typed by PCR were confirmed by the multibead approach, with the exception of 35B, since the multibead assay cannot detect this serotype (39). In addition to the confirmation of isolate serotypes, one additional isolate was identified as type 6C. The CDC was able to identify five isolates as 23A; the remaining 11 isolates were nontypeable.

**MLST.** After concatenation of isolates, we determined that the 11 nontypeable isolates clustered away from the “true” encapsulated pneumococci (Fig. 1). This means that, for each of these isolates, a majority of the seven alleles sequenced were considered to be novel alleles (differing from known alleles in the MLST database by more than 1 to 2%). The remaining 11 strains clustered with true pneumococci and therefore were submitted to determine the sequence types. Upon examination, two isolates were of the same sequence type and, there-
fore, were likely the same isolate. This conclusion was based on the fact that, at the time of isolation, we chose multiple colonies from a single patient swab based on slight differences in morphology. Similarly, another five isolates were also determined to be the same; however, in this case, multiple colonies were chosen from this patient at random to determine if multiple capsule/sequence types would be carried by the same individual. In total, we identified six unique isolates (of the original 22 presumed pneumococci) that were true pneumococci. The results of the MLST analysis are presented in Table 2. Each isolate had a different sequence type, with one of these isolates representing a new type due to the presence of a novel xpt allele. These isolates have been added to the MLST database (http://spneumoniae.mlst.net/).

**Antibiotic susceptibility.** Antibiotic susceptibilities of the pneumococcal isolates were determined for the following antibiotics used in either treatment of pneumococcal infections or for prophylaxis to prevent opportunistic infections in HIV-infected patients: penicillin, moxifloxacin, TMP-SMX, and azithromycin. As expected, all isolates were sensitive to moxifloxacin (Table 2). Only two of the isolates were sensitive to all antibiotics studied; these isolates were carried by patients who were not on any antimicrobial agents (one of them was also not taking antiretrovirals). According to the updated breakpoints for penicillin susceptibility, all of our strains were classified as susceptible (6). Half of the isolates were resistant to azithromycin. Two were resistant to TMP-SMX; one of those strains was isolated from a patient who was currently taking this medication for prophylaxis.

**Carriage.** *S. pneumoniae* was isolated from six patients during this study, providing an overall carriage rate of 3.4%. Four of those patients had CD4+ cell counts of >200/mm³, and two patients had counts of <200/mm³, resulting in carriage rates of about 3% in each group. The average age of carriers was approximately 40 years; most were about 3% in each group. The average age of carriers was 45 years old, while one patient was 64 years old. All carriers were black, but other demographics and/or risk factors were split about evenly (e.g., gender, smoking history, and antibiotic use). Two notable exceptions were that (i) individuals who carried pneumococci, except one, had been vaccinated with PPV23 (which is the same percentage as the entire study population of patients who had ever received the vaccine—82 to 83%) and (ii) only one carrier patient had children younger than 5 years old in the household. Antibiotic use in carriers was basically related to antibiotic use in carriers was basically related to the CD4+ cell count; the two patients with low counts were on prophylactic TMP-SMX. One patient with a higher CD4+ cell count was currently being treated with ampicillin. Season also seemed to play a role in carriage; five of six isolates were collected during fall/winter (October to January), while only one was isolated in the summer (June). This trend may be due to the fact that most of our samples (51.4%) were obtained during the winter months.

**DISCUSSION**

The goal of our study was to examine pneumococcal carriage within an HIV-infected population and to characterize carriage strains. Similar to results in a previous study (32), we saw no correlation between CD4+ cell count and pneumococcal carriage, although the total number of carriers was low. However, compared to the results from the Texas study, our population has a lower overall carriage rate (3.4% versus 12.6%) (32). This decrease in pneumococcal carriage may be related to a number of factors. One reason may be the widespread use of HAART combined with the administration of PPV23 in the routine care of HIV-infected patients, especially early in the disease process. Both of these interventions have been shown to decrease the rate of IPD in this group (7, 10, 30).

A more significant cause may be the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in 2000. Trials studying PCV7 in HIV-infected adults have shown its ability to produce an immunological response in this population, which is often better than the response to PPV23 (8, 20, 23). However, PCV7 is not approved for adults but rather for infants and young children; groups in which PCV7 has been quite effective in protecting against both carriage and invasive disease (5, 25). Since children <5 years old are important reservoirs for pneumococcal carriage, a decrease in pneumococcal carriage and disease in this group will potentially translate into widespread effects on the general adult population (11, 22, 38), as well as HIV-infected individuals (9).

Nonvaccine pneumococcal serotypes are an increasing cause of invasive disease. This phenomenon, known as serotype replacement, is more pronounced in the HIV-infected population (9, 15). In our study, only 1 of the 6 serotypes identified was covered by the conjugate vaccine (Table 2). Some serotypes, such as 11A and 35B are clearly not covered by PCV7, whereas the others (19A, 6C, 23A) may seem to be covered by cross-protection (with 19F, 6B, and 23F, respectively). However, there may be indications otherwise; for example, 6C, a newly identified serotype, is structurally different from 6B, to the point where cross-protection may not be sufficient to prevent carriage or disease (28). Likewise, the incidence of carriage and disease

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**TABLE 2. Characteristics of the pneumococcal clinical carriage isolates from HIV-infected adults**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsule type</th>
<th>Sequence type</th>
<th>Antibiotic susceptibility results fora:</th>
<th>T/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO0</td>
<td>35B</td>
<td>558</td>
<td>S</td>
<td>S/S</td>
</tr>
<tr>
<td>CO10</td>
<td>11A</td>
<td>62</td>
<td>S</td>
<td>S/S</td>
</tr>
<tr>
<td>CO11</td>
<td>19F</td>
<td>177</td>
<td>R</td>
<td>S/S</td>
</tr>
<tr>
<td>CO13</td>
<td>19A</td>
<td>3426</td>
<td>R</td>
<td>S/S</td>
</tr>
<tr>
<td>CO14</td>
<td>6C</td>
<td>1092</td>
<td>S</td>
<td>S/R</td>
</tr>
<tr>
<td>CO18</td>
<td>23A</td>
<td>338</td>
<td>R</td>
<td>S/S</td>
</tr>
</tbody>
</table>


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*AZ, azithromycin; PEN, penicillin; MOX, moxifloxacin; T/S, trimethoprim-sulfamethoxazole; S, susceptible; R, resistant.*
caused by serotype 19A is on the rise due to the lack of cross-reactivity with PCV7 antibodies (34, 40), and it is also associated with multidrug resistance (16, 29). It is worth mentioning that our 19A isolate was also determined to be a new sequence type according to the MLST database, providing more evidence for the ongoing mutations and recombination events that may occur in nonvaccine isolates (4).

Initially, 17 isolates (not including the duplicate isolates) were presumptively identified by commonly used clinical testing methods, such as alpha-hemolysis, optochin sensitivity, and bile solubility. However, 11 of these isolates were determined not to be pneumococci by MLST. This outcome illustrates problems that many clinicians and microbiologists face when trying to properly identify S. pneumoniae and differentiate it from similar streptococcal species (24). Others have used genetic methods to exclude isolates that were presumed to be (and previously reported to be) pneumococci based on standard identification tools (13, 31, 33, 37), with various rates of false positives (ranging from 3.7 to 70.0%) compared to our rate of 64.7%. A common method for genetic confirmation of S. pneumoniae identity is by probing for pneumococccus-specific rRNA sequences (the AccuProbe test), which was used in the previous HIV carriage study (32). While, to our knowledge, there are no direct comparisons between MLST and AccuProbe, we feel that either of these methods should be used to confirm the results of phenotypic tests in epidemiological studies. Evidence shows that the misidentification of pneumococci can affect results of studies looking at antibiotic resistance (31, 37), and no doubt those of many other epidemiological studies.

Although no further testing was done on our nonpneumococcal isolates, they likely fall within the mitis group of streptococci, possibly as a proposed new species, Streptococcus pseudopneumoniae. Not much is known about this organism, but it has been shown to be pathogenic in immunocompetent mice (14), suggesting a pathogenic potential in humans as well. In the literature thus far, the isolates identified as S. pseudopneumoniae candidates have been isolated from patients with some type of invasive disease (1, 19), suggesting a pathogenic potential in this organism in the HIV-infected population, and it will be worthwhile to continue to monitor this organism, especially in light of vaccine-induced strain replacement, which may extend to similar organisms that share the same niche.

Since 1997, there has been a decrease in the pneumococcal carriage rate in HIV-infected adults, which is likely a result of increased efforts to protect the HIV-infected population against IPD and the addition of the pneumococcal conjugate vaccine to childhood immunization recommendations. However, even though overall carriage is decreasing, there is an increase in nonvaccine types, especially in many at-risk populations. Overall, continued use of PCV7 in the pediatric population will be beneficial to the adult HIV-infected population. We should also continue to closely monitor trends to protect against emerging threats to both HIV-infected patients and the population as a whole. Likewise, it is important that those in clinical microbiology consider adding genetic methods to their protocol for identifying S. pneumoniae isolates, especially when reporting on large population-based studies.

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