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BRIEF REPORT

Pulmonary Disease Associated With Nonencapsulated *Streptococcus pneumoniae*

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We discuss 3 patients presenting with pneumonia associated with nonencapsulated *Streptococcus pneumoniae* (NESp), an emerging pathogen commonly causing upper respiratory infections. Clinical isolates obtained from these patients were characterized to evaluate their respective antibiotic resistance and virulence mechanisms. We demonstrate that NESp resistant to classical drug treatments are isolated during pneumonia.

Keywords. AliD; NESp, pneumococcus, pneumonia, PspK.

Streptococcus pneumoniae (pneumococcus) is a bacterium that asymptomatically colonizes the human nasopharynx but also consistently causes diseases such as otitis media and pneumonia [1, 2]. Currently licensed vaccines protect against pneumococcal disease associated with encapsulated strains but elicit no protection against nonencapsulated *S. pneumoniae* (NESp) [3]. Consequently, increased vaccine use has selectively increased isolation of NESp that encode the virulence-associated genes *pspK, aliC,* and *aliD* [4, 5]. Understanding how NESp establish infections is essential to preventing NESp-associated disease. Additionally, analyzing links between patient comorbidities and susceptibility to pneumococcal infection could improve prophylactic patient care. Mississippi has the highest vaccination rates in the United States, at >97.4% coverage [6]. Mississippi also has a high prevalence of chronic diseases, including chronic obstructive pulmonary disease (COPD), hypertension, diabetes, and obesity [7–9]. Due to vaccine-induced selective pressure and high disease rates in Mississippi, the aim of this study was to determine NESp prevalence among Mississippians presenting with pneumococcal disease and determine the virulence mechanisms associated with isolated NESp.

METHODS

Clinical Isolates

Thirty-five *S. pneumoniae* clinical isolates were retrieved from the University of Mississippi Medical Center (UMMC) Microbiology Surveillance Lab (Jackson, MS) between October 2015 and July 2016. *S. pneumoniae* identification was verified by alpha hemolysis and optochin sensitivity. The previously characterized UMMC NESp clinical isolate C144.66 was included in this study for comparison purposes [10]. Antimicrobial susceptibility testing of bacterial cultures using VITEK 2 was completed by the UMMC microbiology clinical lab. Patient characteristics, sources of infection, and relative antibiotic resistance are listed in Table 1.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification was performed with primers for the conserved capsule gene *cpsA* (forward: 5'-GCAGTACAGCAGTTTGTTGGACTGACC-3' and reverse: 5'-GAATATTTTCATTATCAGTCCCAGTC-3') or nonencapsulated genes *pspK* (forward: GCAAATCAGCCAGTAACTGTGA-3' and reverse: 5'-CAAGATAAGCTTTCTGCACCTCT-3'), *aliC* (forward: 5'-GACCAGATTACCAAGATCCAGCAAC-3' and reverse: 5'-GCCCTTTGTTATACCTAGATGTTTC-3'), and *aliD* (forward: 5'-AGATGCCAAATGGTTCACGGCA-3' and reverse: 5'GGTCGTCAATGGCCTTCACC-3'). The encapsulated pneumococcal strain WU2 was used as a positive control for *cpsA* amplification. NESp strains MNZ41 ($aliC^+$ $aliD^+$) and MNZ67 ($pspK^+$) were used as positive controls for amplification of *aliC*, *aliD*, and *pspK*. PCR products were amplified using GoTaq (Promega) with the cycling parameters suggested by the manufacturer and an annealing temperature of 52°C. Amplified PCR products were analyzed on a 1% agarose gel with ethidium bromide staining.

Biofilm Formation

A 24-well plate was seeded with 10^5 colony forming units (CFU) pneumococci in biofilm media (Todd-Hewitt broth with 0.5% yeast extract, 8 U/mL catalase, and 10% horse serum) per well and incubated at 37°C with 5% CO_2 for 24 hours. Following incubation, biofilm media was removed from the wells, and biofilms were

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Table 1. Clinical Correlation and Characteristics of NESp Isolated From Patients

CI	Source	Age/Sex	Presentation/Comorbidity	Immunization	CA vs HA	Antibiotic Resistance	Virulence Gene Encoded
C144.66	Middle ear	2/M	Chronic adenoiditis	ND	CA	Erythromycin Penicillin Sulfamethoxazole/trimethoprim	*pspK*
361.67	Pulmonary	48/M	Intubation ARF	PPSV 2015	HA	Clindamycin Tetracycline Erythromycin	*pspK*
327.251	Pulmonary	66/M	COPD Diabetes	PPSV 2013 and 2016	CA	Penicillin	*aliD*
264.174	Pulmonary	5/F	Cystic fibrosis	Not immunized	CA	Erythromycin	*aliD*

Abbreviations: ARF, acute respiratory failure; CA, community-acquired; CI, clinical isolate; COPD, chronic obstructive pulmonary disease; HA, hospital-acquired; ND, not determined; NESp, nonencapsulated *Streptococcus pneumoniae*; PPSV, pneumococcal polysaccharide vaccine (Pneumovax23).

stained with 350 µL of 0.1% crystal violet at room temperature for 30 minutes. Unbound crystal violet was carefully removed, 1 mL of 100% ethanol was added to each well to solubilize biofilm-associated crystal violet, and the plate was shaken at room temperature for 10 minutes. Solubilized crystal violet was transferred to a 96-well plate that was measured at OD_{630} with an xMark microplate spectrophotometer (BioRad). All samples were assayed in triplicate, and independent experiments were performed twice.

Pneumococcal Adherence and Invasion of Epithelial Cells

Human Detroit 562 pharyngeal and A549 pulmonary epithelial cells were grown to approximately 90% confluence in 10% fetal calf serum (FCS)–supplemented Eagle's minimal essential medium (EMEM) with 100 µg/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL amphotericin B in 24-well plates at 37°C with 5% CO_2. Pneumococcal adherence and invasion assays were performed as previously described [11]. Briefly, 10^7 pneumococci suspended in antibiotic-free EMEM was added to each well. Adhered pneumococci were enumerated after 30 minutes of incubation with human epithelial cells. To assess invasion, pneumococci were incubated with epithelial cells for 2 hours, followed by elimination of extracellular bacteria with 200 µg/mL gentamycin before lysing epithelial cells and enumerating intracellular pneumococcal CFUs. Two independent experiments were performed, and all samples were assayed in triplicate.

Statistical Analysis

PRISM 5 software (GraphPad Software, Inc.) was used to analyze the results. To assess if there were any significant differences in the means between all groups compared, we used one-way analysis of variance (ANOVA). Tukey post-tests allowed us to identify the specific significant differences in the groups analyzed. A *P* value of less than .05 was considered statistically significant.

RESULTS

Survey of Pneumococcal Isolates and Clinical Correlation

PCR analysis of the capsule locus demonstrated that 3/35 (~9%) pneumococcal isolates lacked the highly conserved capsule gene *cpsA*. Further PCR analysis revealed that 361.67 encoded *pspK* whereas 327.251 and 264.174 encoded *aliD* (Table 1). All 3 newly identified NESp isolates were associated with pulmonary disease (Table 1); 361.67 was associated with acute pneumonia in an adult male following intubation during acute respiratory failure, and 327.251 was isolated during a COPD exacerbation in a 66-year-old male. Both adult male patients were previously vaccinated against pneumococcus (Table 1); 264.174 was isolated from a cystic fibrosis (CF) pediatric patient who had received no prior vaccination against pneumococcus. The "gold standard" treatment for *S. pneumoniae* infections is penicillin, and 2 out of the 4 strains were found to be resistant to penicillin (Table 1). NESp strains were also resistant to antibiotics commonly used to treat *S. pneumoniae* infections, such as erythromycin and sulfamethoxazole/trimethoprim.

NESp Phenotypes Permit Persistence in a Host

PspK is an adhesin that aids in adherence to host cells, and AliC and AliD are oligopeptide binding proteins that sense the extracellular environment [5]. We grouped isolates based on expression of PspK or AliD to examine virulence mechanisms. Human pharyngeal and pulmonary epithelial cells were used to mimic the host niche during colonization and pneumonia (Figure 1). All clinical isolates efficiently adhered to pharyngeal cells (Figure 1A). PspK-expressing 361.67 adhered to A549 pulmonary cells significantly more ($P < .001$) than all other isolates (Figure 1B). PspK-expressing cells invaded both pharyngeal and pulmonary cells significantly more ($P < .001$) than their AliD-expressing counterparts (Figure 1C and D). When comparing epithelial cell invasion by PspK-expressing strains C144.66 and 361.67, C144.66 demonstrated significantly greater ($P < .01$) invasion of pharyngeal cells (Figure 1C), whereas 361.67 demonstrated significantly greater ($P < .05$) invasion of pulmonary cells (Figure 1D). All NESp strains formed dense biofilms (Figure 1D), but the AliD-expressing strain 327.251 formed significantly more biofilm than the other pulmonary isolates 361.67 ($P < .05$) and 264.174 ($P < .01$).

DISCUSSION

In this report, we determined that nearly 9% of pneumococcal infections at UMMC were linked to NESp, and we described

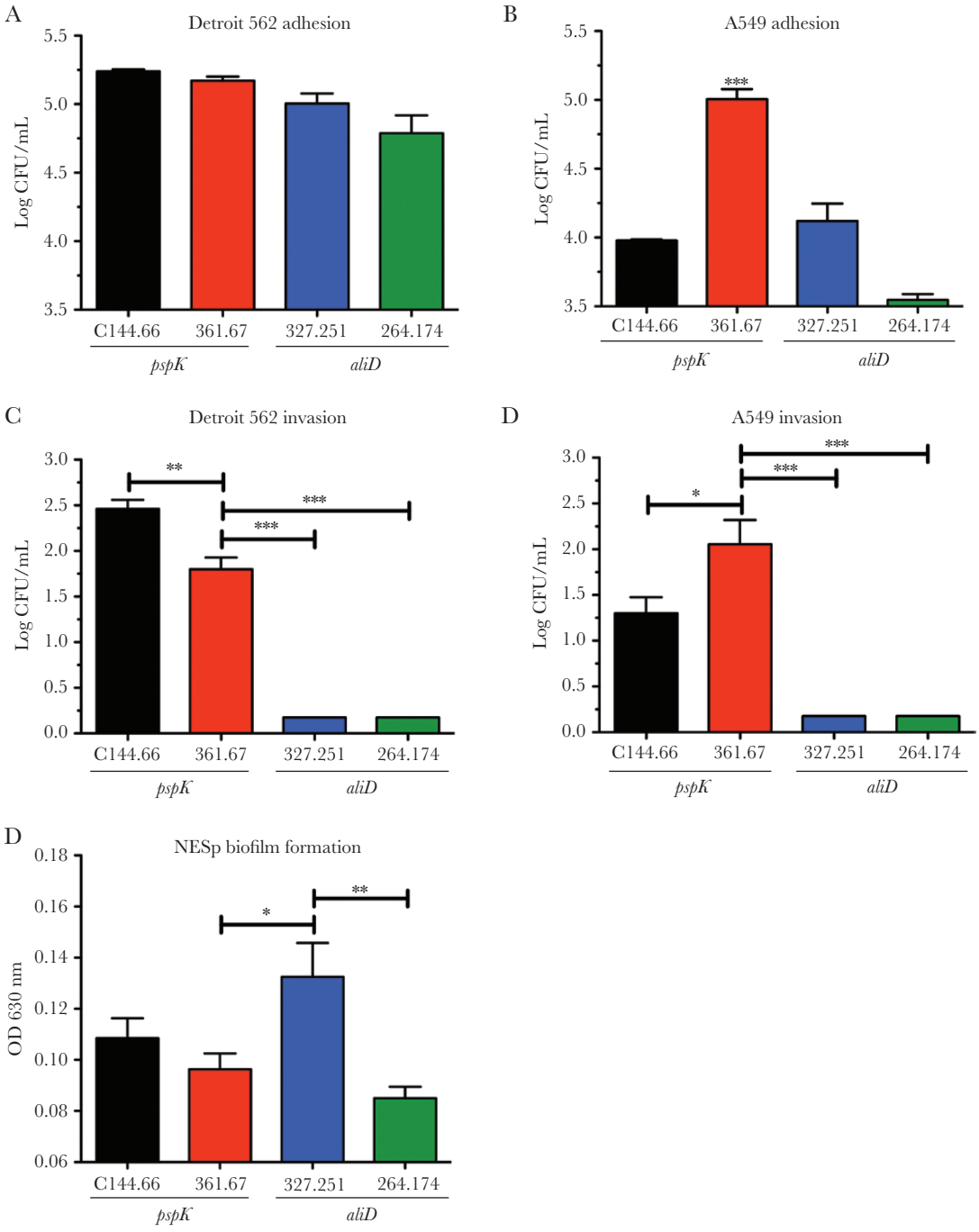

Figure 1. Nonencapsulated *Streptococcus pneumoniae* (NESp) phenotypes influencing pulmonary disease. Human Detroit 562 pharyngeal (A and C) and A549 pulmonary (B and D) epithelial cells were used to assess NESp adherence (A and B) and invasion (C and D). Biofilms formed (E) after 24-hour growth in 24-well plates were assayed using crystal violet staining and measuring the optical density at 630 nm. A, All clinical isolates efficiently adhered to pharyngeal cells. B, Clinical isolate (CI) 361.67 adhered to pulmonary cells significantly more than all other isolates. PspK-expressing cells invaded both pharyngeal (C) and pulmonary (D) cells at low rates. CI C144.66 invaded pharyngeal cells more efficiently than 361.67 (C), whereas 361.67 invaded pulmonary cells more efficiently than C144.66 (D). CI 327.251 formed significantly more biofilm than 361.67 or 264.174. Data represent 2 independent studies performed in triplicate. Error bars denote standard error of the mean ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$). Abbreviations: CFU, colony forming units; OD, optical density.

3 patients who develop NESp-associated pneumonia. We also demonstrated that NESp express antibiotic-resistant phenotypes that threaten classical treatment measures. As there are no currently licensed NESp preventatives, antibiotic-resistant NESp are an emerging threat to public health. To better understand how NESp are able to establish disease, we examined virulence mechanisms of the clinical isolates. All NESp isolates efficiently adhered to pharyngeal cells and formed dense biofilms, which likely aided in nasopharyngeal colonization and development of pneumonia. NESp did not invade epithelial cells efficiently,

which suggests that NESp are able to withstand the extracellular environment despite the lack of a protective polysaccharide capsule.

In the patient infected with 361.67, translocation of 361.67 to the lungs during intubation could have led to the infection, which is a phenomenon that has been previously reported [12]. As PspK has been shown to increase adherence to pulmonary epithelial cells and persistence in a murine lung model, PspK of 361.67 may have also facilitated persistence during pulmonary infection [11]; 361.67 had greater adherence to A549 pulmonary cells when compared with a clinical isolate also expressing PspK (C144.66), which may have been due to diverse genetic backgrounds or differential expression patterns that increase interactions with epithelial cells. C144.66 also invaded pharyngeal cells significantly more than 361.67 and caused chronic adenoiditis rather than pneumonia. Even though both these strains express PspK, NESp may have a predilection for certain anatomic and physiologic environments. NESp strains isolated during conjunctivitis encode niche-specific adaptations, and similar adaptations likely occur during middle ear or lung infections [13]. NESp strains expressing AliD showed no invasive potential in vitro but caused pulmonary infections in the clinical setting. The altered lung environment in COPD and cystic fibrosis patients may be potentiating these NESp infections by supplying an altered host niche that supports NESp growth; for instance, chronic hypersecretion of mucus and air trapping that prevents proper expansion of the lungs predispose this site to infection [14]. Also, invasive pneumococcal disease caused by encapsulated strains has been shown to be more prevalent in patients with a chronic pulmonary condition, and this is likely the case for NESp as well [15]. Pneumococcal vaccinations are currently recommended for patients who are immunosuppressed or diagnosed with chronic pulmonary diseases. Vaccinating these individuals reduces the risk of pneumococcal disease associated with vaccine-covered serotypes but increases the risk of NESp-associated disease in these patients. Overall, NESp strains are becoming more prominent human pathogens, and patients with certain comorbid conditions are at a higher risk of diseases associated with these strains. Further surveillance of NESp isolates will need to be conducted to determine the widespread NESp threat to public health and NESp-specific virulence mechanisms.

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Conflicts of interest. None declared.

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