Nonencapsulated *Streptococcus pneumoniae* Cause Acute Otitis Media in the Chinchilla That Is Enhanced by Pneumococcal Surface Protein K

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Background. Use of the pneumococcal conjugate vaccine has led to serotype replacement of carriage and acute otitis media (AOM) pneumococcal isolates. Increases in nonencapsulated *Streptococcus pneumoniae* (NESp) isolates have also occurred, and there are increasing reports of NESp-associated disease. Disease prevalence and virulence factors of NESp isolates have not been studied.

Methods. A chinchilla model of pneumococcal AOM was utilized, and disease was assessed through bacterial enumeration along with scoring visible signs of pathology. An adhesion-invasion assay using a human epithelial cell line was performed.

Results. Nonencapsulated *Streptococcus pneumoniae* strains containing pneumococcal surface protein K (PspK) were more likely to cause AOM and pathology upon infection. Deletion of PspK from an isolate significantly reduced bacterial loads. Increased epithelial cell adhesion correlated with increased virulence of NESp isolates naturally lacking PspK. Furthermore, expression of PspK by an avirulent NESp resulted in virulence.

Conclusions. The presence of PspK increased the disease potential of NESp. Pneumococcal surface protein K is not the only virulence factor of NESp in AOM. Expression of PspK in an avirulent NESp mediated the progression to pneumococcal disease. Genetic exchange between pneumococci may allow dissemination of PspK, increasing the potential of NESp disease. The current study is the first report of a NESp-specific virulence factor.

Keywords. acute otitis media; AOM; NESp; nonencapsulated *Streptococcus pneumoniae*; pneumococcal surface protein K; PspK.

Disease burden caused by *Streptococcus pneumoniae* (the pneumococcus) is highest in children under the age of 5 [1]. The most common cause for children under 5 to visit a physician and receive antibiotics is acute otitis media (AOM) [2, 3]. The pneumococcus is the leading cause of bacterial AOM, followed by *Haemophilus influenzae* and *Moraxella catarrhalis*, respectively [4]. A prerequisite for pneumococcal disease is the establishment of nasopharyngeal colonization, after which pneumococci can disseminate into normally sterile sites [5]. It has been shown that nonencapsulated *S pneumoniae* (NESp) colonizes the human nasopharynx and that the NESp-specific pneumococcal surface protein K (PspK) aids in colonization [6, 7]. We define NESp as being pneumococcus based on multilocus sequence typing and lacking the ability to synthesize capsule polysaccharide. Previous reports of nontypeable pneumococcus are based on an inability to identify serotype, which does not confirm absence of capsule. Therefore, we suggest NESp terminology to increase clarity of capsule status.

Introduction of the pneumococcal protein-polysaccharide conjugate vaccine (PCV) has not significantly reduced the rates of pneumococcal carriage because nonvaccine serotypes have increased prevalence in the population, compensating for reduced carriage of vaccine serotypes [8, 9]. There is also an increased incidence

of pneumococcal disease caused by serotypes not contained within the PCV [10, 11]. Nonencapsulated *S pneumoniae* lack capsule and are not targeted by the currently available vaccines, which may have contributed to the increase in NESp carriage [12]. A recent report indicated as much as 16% of the pneumococcal isolates in an unvaccinated population do not express capsule, which may be higher in a vaccinated population [13]. An extensive survey of pneumococcal invasive isolates found that 0.61% of these isolates were NESp [14]. Although this is a small percentage, it shows that NESp can cause systemic disease that cannot be protected against by the current vaccine. This increase in colonization and dissemination of NESp in the human population increases the risk of pneumococcal disease caused by NESp. Thus, NESp may be a newly emerging human pathogen.

It has previously been shown that PspK is required for colonization of the mouse nasopharynx by a subset of NESp, and we have shown that PspK increases adhesion to human epithelial cells [6, 7]. Other pneumococcal surface proteins that increase adhesion to epithelial cells have been shown to increase pneumococcal virulence [15–18]. Pneumococcal surface protein K of NESp, which also increases epithelial cell adhesion, may also perform a role in virulence. Nonencapsulated *S pneumoniae* are more likely to cause noninvasive diseases, such as AOM and sinusitis, due to the lack of protection afforded by the capsule in the blood [7]. The adhesive properties of PspK need further investigation in the role of disease to understand the potential of NESp-specific factors in disease progression.

The model organism for testing bacterial AOM is the chinchilla due to its easily accessible bulla and physiological similarities to the human bulla [19, 20]. To date, there has been 1 human case of AOM caused by NESp that has been reproduced in the chinchilla model [21]. Others have reported both invasive and noninvasive pneumococcal diseases caused by NESp, but these isolates were not tested in animal models [22–25]. The current study tests the ability of a genetically diverse group of the newly emerging human pathogen NESp to cause AOM in a chinchilla model. The effect of PspK on disease and the role of adhesion in virulent strains were also examined.

METHODS

Bacteria

Pneumococcal strains were grown at 37°C in 5% CO_2 either on sheep blood agar (BA) or in Todd-Hewitt medium with 0.5% yeast extract (THY) with appropriate antibiotic selection. Strains used in this study are described in Table 1.

Genetic Manipulation

MNZ67 isogenic PspK mutant was made by allelic replacement of the capsule locus with a DNA fragment containing a spectinomycin resistance cassette flanked by the pneumococcal genes *aliA* and *dexB* as previously described [6, 26]. In brief, pneumococci were transformed in competence medium with bacteria stimulated by 200 ng of competence-stimulating peptide 1 (CSP1) and selected on BA spectinomycin (300 µg/mL) to produce LEK05 (MNZ67 ΔPspK).

For expression of PspK in pneumococci, we used the previously constructed plasmid pNE1::PspK [7]. Pneumococci were

Table 1. Pneumococcal Strains and Clinical Scores 4 Days Postinfection

Strain	PspK	Source of Isolate	Sequence Type	Associated Serotype	Otic Score (Mean ± SE)[*]	Biofilm Score (Mean ± SE)[*]	Reference
B1351	–	Conjunctivitis	448	NESp	0.00 ± 0.00	0.00 ± 0.00	This Study
MNZ14	–	Carriage	448	NESp	0.50 ± 0.29	0.00 ± 0.00	[6]
MNZ41	–	Carriage	6153	NESp	0.63 ± 0.18	2.25 ± 0.16	[6]
MNZ85	–	Carriage	2315	NESp	1.75 ± 0.25	0.25 ± 0.25	[6]
AW124	–	Carriage	5201	Type 4	1.00 ± 0.00	1.5 ± 0.25	[24]
R36A	–	D39 capsule mutant	595	NESp	0.67 ± 0.33	0.60 ± 0.37	[37]
LEK01	+	R36A expressing PspK	595	NESp	1.50 ± 0.22	1.20 ± 0.34	[7]
C144.66	+	Adenoiditis	9370	NESp	1.00 ± 0.27	1.13 ± 0.35	This Study
MNZ11	+	Carriage	6151	NESp	1.00 ± 0.58	2.00 ± 0.58	[6]
MNZ15	+	Carriage	1106	Type 14	1.25 ± 0.31	0.63 ± 0.26	[6]
MNZ24	+	Carriage	6152	NESp	1.75 ± 0.25	0.5 ± 0.29	[6]
MNZ37	+	Carriage	1106	Type 14	0.75 ± 0.25	1.75 ± 0.25	[6]
MNZ50	+	Carriage	1106	Type 14	0.88 ± 0.23	0.88 ± 0.30	[6]
MNZ66	+	Carriage	1106	Type 14	1.25 ± 0.48	0.75 ± 0.48	[6]
MNZ67	+	Carriage	1464	Type 19F	1.00 ± 0.41	2.00 ± 0.41	[6]
LEK05	–	MNZ67 PspK deletion mutant	1464	Type 19F	0.00 ± 0.00	0.25 ± 0.25	This Study

Abbreviations: NESp, nonencapsulated *Streptococcus pneumoniae*; PspK, pneumococcal surface protein K; SE, standard error.

[*] Contains only day 4 data because there was no significant difference between day 4 and 7 pathology in tested strains.

transformed with pNE1::PspK in competence medium with bacteria stimulated by 200 ng of CSP1. Pneumococci were incubated with 1 μg of DNA for 4 h and plated on BA with 300 μg/mL spectinomycin. Single colonies were isolated and grown in THY containing 300 μg/mL spectinomycin and stored at −80°C after the addition of 20% glycerol.

Expression of PspK in vivo was tested by flow cytometry as described previously [7]. In brief, rabbit polyclonal anti-PspK serum was used to stain samples and visualized with an antirabbit biotinylated secondary and streptavidin-conjugated Alexa Fluor 488 followed by flow cytometry analysis.

Experimental AOM

A chinchilla otitis media model was used to assess the ability of NESp to cause AOM. Young adult chinchillas (*Chinchilla lanigera*, body weight 400–500 g) from Ryerson Chinchilla Ranch were allowed to acclimate for at least 7 days. An otoscope was used to examine the tympanic membrane of all animals before infection. Studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Both the left and right bullae were inoculated via transbullar injection of $\sim 10^7$ bacteria suspended in 100 μL phosphate-buffered saline (PBS; pH 7.2) containing 0.04% gelatin [27]. All inocula were confirmed by plating on BA. A minimum of 4 bullae was infected for each isolate tested at the specified time point.

Chinchilla were sacrificed at the indicated time postinfection and otic score assessed. Tympanic pathology scored through otoscopic examination as follows: 0 = none, 1 = inflammation, 2 = effusion, and 3 = tympanic rupture. The skin was reflected from the top of the head to expose the bullae. Access to the middle ear was achieved by cutting a 1 cm diameter hole in the bullae. Biofilm formation was visibly scored as follows: 0 = none, 1 = surface formation, 2 = traverses bulla, and 3 = traverses bulla with thickening. Middle ear effusion (MEF) was collected and each bulla was lavaged with 1 mL PBS. These samples were used to determine the numbers of bacteria not associated with biofilm. The bullae were homogenized in 10 mL PBS and used to estimate the surface-associated community bacteria. All samples were enumerated on BA with 5 μg/mL gentamicin.

Adhesion Invasion Assay

An adhesion and invasion assay was performed as described previously [7]. In brief, Detroit 562 cells, a human nasopharyngeal epithelial cell line, were grown to ~95% confluence in 24-well culture plates. Epithelial cells were inoculated with 10^7 bacteria and incubated for 30 minutes. The number of adherent bacteria was estimated on BA after trypsinization of epithelial cells. Epithelial cell invasion was quantitated by 2 hour incubation with 10^7 bacteria followed by an additional 1 hour incubation with media containing penicillin (10 μg/mL) and gentamicin (200 μg/mL). Epithelial cells are then lysed and bacteria enumerated on BA.

Data expressed as percentage of adherent bacteria standardized to MNZ11 for adherence and MNZ41 for invasion set at 100%. Each experiment had at least 3 biological replicates for each strain.

Statistical Analysis

Colony-forming units (CFUs) in different experimental groups were compared using the Student's *t* test with Prism 5 software (GraphPad Software, Inc., San Diego, CA). Correlation analysis was performed with Prism 5 software by linear regression analysis.

RESULTS

PspK-Deficient NESp AOM

Six wild-type NESp isolates of varying sequence types (STs) that naturally lack *pspK* were tested for the ability to cause AOM in a chinchilla model. The strains tested were MNZ14, MNZ41, MNZ85, B1351, AW124, and R36A (Table 1). MNZ41, MNZ85, and AW124 were able to maintain a bacterial load in the bullae at the times tested and showed increased signs of tympanic pathology and biofilm formation compared with the other strains tested (Figure 1A and C). Only B1351 showed no visible pathology (Table 1).

PspK+ NESp AOM

Eight wild-type PspK+ strains were tested: MNZ11, MNZ15, MNZ24, MNZ50, MNZ66, MNZ67, MNZ37, and C144.66 (Table 1). All 8 of these PspK+ NESp strains were able to cause AOM in the chinchilla model at 4 days postinfection. Of those strains tested at 7 days postinfection, there was no significant difference from bacterial loads observed at day 4 as determined by bacterial enumeration (Figure 1B and D) and clinical signs of AOM (Table 1). Although all the PspK+ isolates could cause AOM, the number of bacteria in the bulla varied among the different isolates. Genetic background based on ST was independent of the ability to cause disease (Table 1). All 8 PspK+ isolates produced biofilm (Figure 1F) and MEF, and the tympanic membranes showed visible signs of pathology.

Adhesion Invasion Assay

Pneumococcal adhesion and invasion on epithelial cells was assessed to determine whether increased adhesion or invasion correlates with the ability to cause AOM. Of the isolates that did not have PspK (Table 1), the 3 strains that had recoverable bacteria during AOM (MNZ41, MNZ85, and AW124) had significantly increased adhesion ($P < 0.05$) compared with other tested strains (Figure 2A). The exception was MNZ85, which did not have significantly more adhesive cells than MNZ14, but it also had the least recoverable bacteria during AOM (Figure 1A). An increase in biofilm formation and otic pathology are also noted from PspK− NESp with recoverable amounts of bacteria (Table 1). Adhesion of all PspK+ isolates varied considerably with no discernible preference for increased adhesion leading to increased bacterial loads (Figure 2B). In addition,
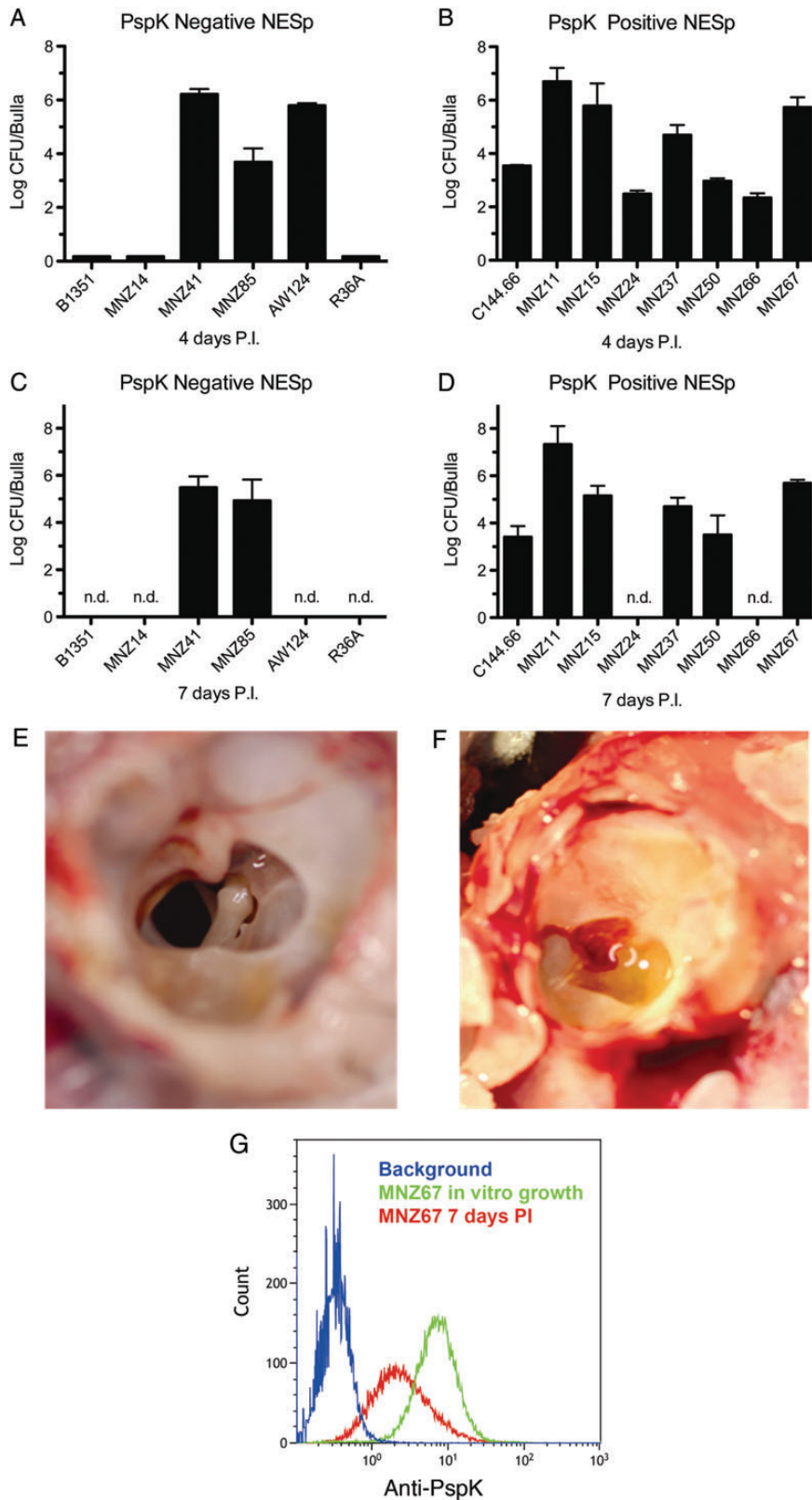
Figure 1. Intrabullar challenge of chinchilla with nonencapsulated *Streptococcus pneumoniae* (NESp). Groups of animals were infected with 1×10^7 pneumococci, and the colony-forming unit (CFU) of pneumococci in the bulla (log CFU/bulla ± standard error of the mean) was determined at 4 and 7 days postchallenge. Data are cumulative from at least 2 independent bulla from 2 animals. (A and C) Challenge with NESp that naturally lack pneumococcal surface protein K (PspK), and (B and D) challenge with PspK⁺ NESp. Strains not determined (n.d.) at 7 days are indicated on graph. Middle ear chambers at 4 days postinfection (E) mock-infected ear and (F) ear infected with MNZ67 PspK⁺ showing biofilm. (G) Strain MNZ67 stained with anti-PspK showing that PspK is surface expressed 7 days postinfection (red) compared with background (blue) and growth in 0.5% yeast extract ([THY]; green).
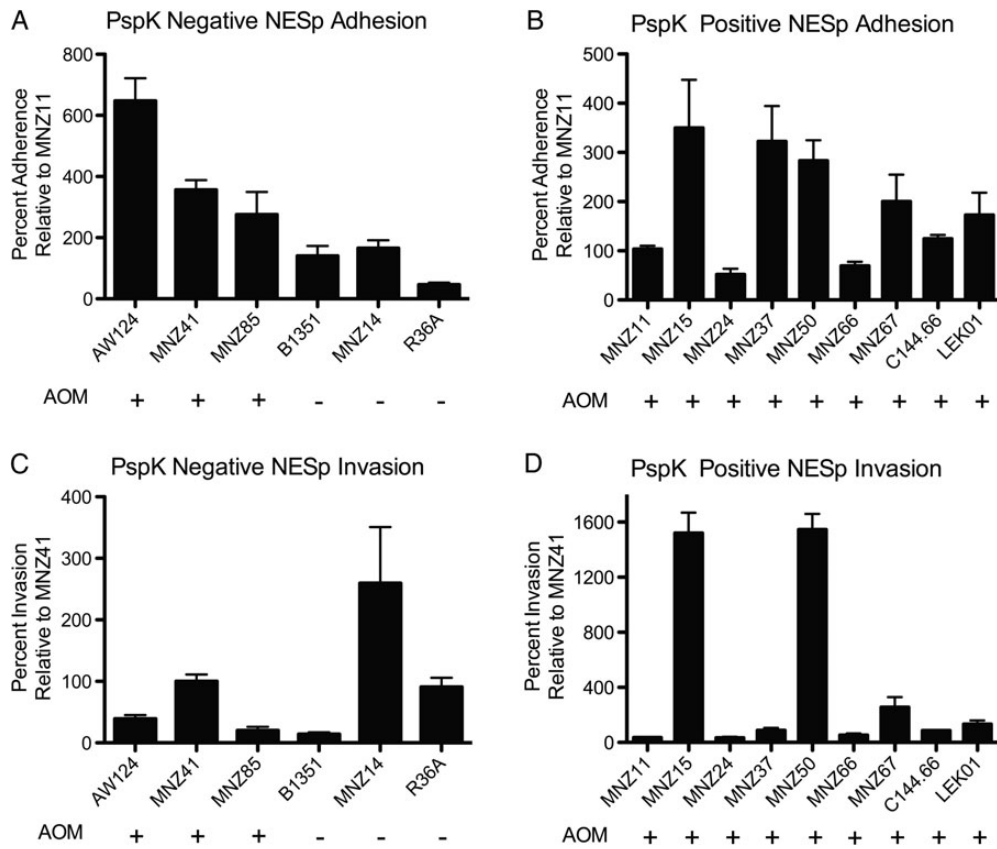
Figure 2. Adherence and invasion of nonencapsulated *Streptococcus pneumoniae* (NESp) isolates on human epithelial cells. Detroit 562 nasopharyngeal epithelial cell line was challenged with NESp, and adherence was determined at 30 minutes, whereas invasion was determined at 2 hours postinoculum followed by antibiotic treatment. (A and B) Adherence expressed as percentage pneumococci adhered to epithelial cells normalized to MNZ11 set at 100% binding. (C and D) Nonencapsulated *S pneumoniae* invasion of epithelial cells expressed as percentage invasion normalized to MNZ41 set at 100% invasion. Values were obtained from triplicate wells infected with the indicated NESp. Acute otitis media (AOM) + indicates those isolates that had recoverable counts in Figure 1, whereas − indicates isolates for which no bacteria were recovered in Figure 1.

no significant correlation was observed when comparing adhesion with the otic and biofilm scores for PspK⁺ NESp.

A significant correlation through linear regression analysis comparing recovered bacteria and adhesion is observed when all strains are compared ($P < 0.05$) or when only PspK⁻ isolates are compared ($P < 0.05$), but not if the analysis includes only PspK⁺ isolates ($P > 0.05$). In contrast to the observed effects of adhesion to epithelial cells, the amount of invasion of PspK⁻ NESp did not correspond to increases in the ability to cause AOM or pathology. Also, no difference in virulence was observed for PspK⁺ isolates based on invasiveness of epithelial cells. There is significant variability in the invasiveness of these isolates, but the most highly invasive isolates in cell line invasion experiments, MNZ15 and MNZ50, vary in AOM pathology and recoverable bacteria during AOM.

AOM mediated by PspK

We have previously demonstrated that PspK increases epithelial cell adhesion, and isogenic PspK mutants are unable to colonize the mouse nasopharynx [6, 7]. The MNZ67 PspK mutant LEK05 was significantly less virulent with respect to numbers of bacteria recovered at both 4 and 7 days postinfection ($P < 0.0001$) (Figure 3), and all signs of pathology were reduced (Table 1).

Pneumococcal strain R36A, a laboratory-derived nonencapsulated D39 (serotype 2), does not effectively colonize the mouse nasopharynx. We have previously reported that LEK01 (R36A expressing PspK) has enhanced epithelial cell adherence and colonization in the mouse [7]. The expression of PspK in R36A significantly increased the number of bacteria in the bullae and resulted in increased pathology and biofilm formation ($P < 0.0001$) (Figure 4).

Pathology of NESp AOM

Otic scores and biofilm formation were visually quantified in each experiment. Table 1 summarizes pathological scores for each isolate tested. Biofilm formation correlates with increased bacterial loads, with more biofilm associated with higher
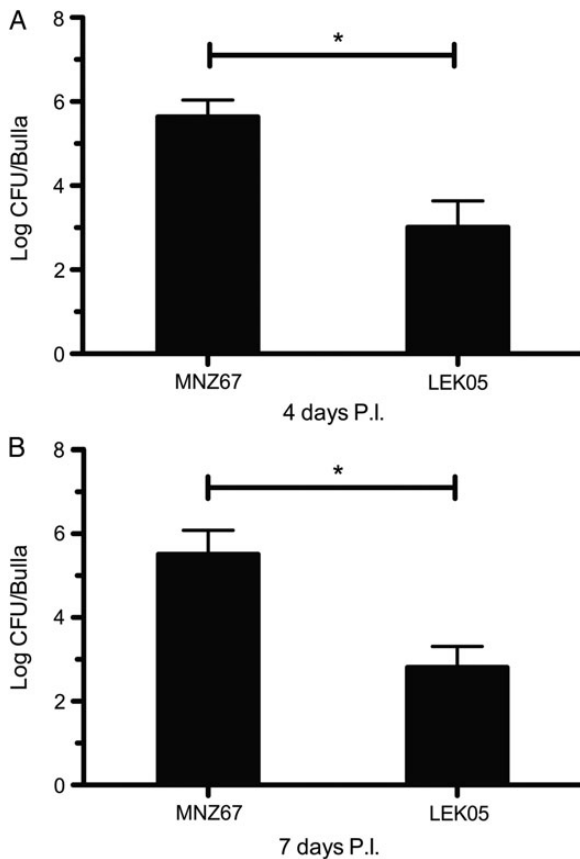
Figure 3. Deletion of pneumococcal surface protein K (PspK) reduces acute otitis media caused by nonencapsulated *Streptococcus pneumoniae* (NESp). After intrabullar challenge, PspK isogenic mutant LEK05 had reduced bacterial burden compared with wild-type MNZ67. Counts displayed as log colony-forming unit/bulla ± standard error. Data represent cumulative data of 2 independent bullae from 2 animals. *, $P < .0001$ by Student's t test.

Figure 4. Pneumococcal surface protein K (PspK) increases pneumococcal virulence in acute otitis media. Four days after intrabullar challenge, LEK01 (R36A PspK$^+$) significantly increased in numbers compared with the parent strain R36A. Counts displayed as log colony-forming unit/bulla ± standard error. Data represent cumulative data of 2 independent bulla from 2 animals. *, $P < .0001$ by Student's t test.

bacterial loads ($P < 0.0005$) (Figure 1E and F). Although increases in tympanic pathology trend to greater bacterial loads during AOM, it does not significantly correlate to increased bacterial loads. Table 1 contains only data from day 4 postinfections because there was no significant difference between day 4 and 7 pathology.

DISCUSSION

The pneumococcus is the most common cause of bacterial AOM [4]. Vaccination efforts against the pneumococcus have greatly reduced invasive pneumococcal disease, but it has not been as effective against reducing colonization and AOM due to serotype switching [8, 9]. Nonencapsulated *S. pneumoniae* can efficiently colonize the human nasopharynx, but it must compete against other pneumococci [6, 28]. Selective pressure against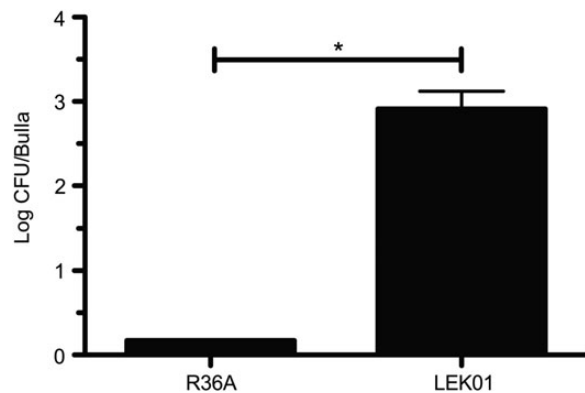 pneumococcal capsular serotypes contained in the vaccine has reduced competition, allowing for an increase in NESp in the human population to occur [12].

Several reports of NESp disease causing both invasive and noninvasive disease have been documented in the literature [14, 22–25]. Reports have indicated a rise in NESp colonization within the human population. This colonization is prerequisite for pneumococcal disease, and the rise in NESp in the population increases the potential of NESp disease. In the current study, we determined that genotypically diverse NESp were able to cause AOM in the chinchilla.

Pneumococcal surface protein K increased disease progression of NESp in this AOM model. Deletion of *pspK* did not abolish the ability of an isolate to cause AOM but did significantly reduce pathology and bacterial counts recovered during an infection. More importantly, expression of PspK by R36A, which was unable to cause AOM, was sufficient to establish AOM. Three of six NESp isolates that naturally lack PspK were able to cause AOM, most likely due to enhanced epithelial cell adhesion compared with the other PspK$^-$ NESp isolates.

We have previously established that PspK aids in adhesion to epithelial cells [7]. Some of our NESp isolates that naturally lack PspK caused AOM, and the increased adhesion to epithelial cells by these NESp may explain their disease causing ability. A significant correlation from linear regression analysis comparing the average log CFU recovered during AOM and percentage adherence to epithelial cells was found ($P < 0.05$). This correlation only applies to isolates that do not contain PspK, indicating that the ability of PspK$^-$ NESp to cause disease is dependent on high levels of adherence. The variability seen in the recovered CFU among PspK$^+$ isolates seems due to other pneumococcal factors, possibly those contained within pathogenic isolates of PspK$^-$ NESp.

The pneumococcus has several surface molecules that increase adhesion, some contained within transferable pathogenicity islands [29]. Two common pathogenicity islands found within encapsulated pneumococcal isolates linked to AOM are pilus islet-1 and 2 (PI-1 and PI-2, respectively) [30, 31]. Genomic analysis of MNZ11, MNZ14, MNZ37, MNZ41, and MN85 reveal neither PI-1 nor PI-2, suggesting other mechanisms resulting in varied pathogenesis [32]. Another important aspect of the disease process is the presence of biofilm in infected chinchillas [27]. Increased amounts of biofilm also correlated with higher bacterial loads recovered during AOM, which was seen in all strains tested. This finding is in contrast to some reports that indicate no differences in the expression of biofilm and the ability to cause disease in humans [33].

CONCLUSIONS

All PspK+ isolates tested, including LEK01 (R36A PspK+), were able to cause disease in our model. R36A is nonencapsulated, which results in avirulence, but PspK expression abrogates the loss of virulence in R36A. Thus, PspK was sufficient to cause disease. Although there are other unknown factors that enhance the disease process, the acquisition of PspK enhances virulence. Pneumococcal surface protein K increases adhesion to epithelial cells, which may explain how the PspK+ isolates were able to cause disease. Polymicrobial pneumococcal populations within the nasopharynx are common, and genetic exchange can occur in the nasopharynx between different pneumococcal strains [34, 35]. If this genetic exchange occurs within the current environment of pneumococcal vaccination, there will be continued pressure against encapsulated strains of serotypes covered in vaccines potentially leading to increases in NESp. The capsule locus of the pneumococcus undergoes genetic recombination and mutation at a high rate, which increases the chance of PspK integration within the capsule locus [36]. Pneumococcal disease cannot occur without efficient nasopharyngeal colonization. Pneumococcal surface protein K expression enhances colonization that potentiates disease by PspK containing NESp [5, 7].

In the chinchilla, NESp can cause AOM with significant pathology and bacterial loads. Furthermore, this is not limited to specific STs but is possible from a genetically diverse population of NESp. All carriage isolates tested in the current study were resistant to at least 1 antibiotic, with erythromycin being the most common (data not shown). A significant proportion of NESp in the population can cause AOM in the chinchilla, and with increases in the prevalence of antibiotic resistance, treatment of the disease will become increasingly difficult. Vaccine induced pressure against the pneumococcal capsule locus propagates mutations in this site leading to either serotype replacement or loss of capsule, which can be replaced with the virulence factor PspK. We demonstrated that PspK acquisition results in disease in the chinchilla. Thus, acquisition of PspK by pneumococci in the human population could increase the potential for NESp disease. We propose that increases in NESp and dissemination of virulence factors, such as PspK and antibiotic resistance, potentiates the possibility of noninvasive diseases by the newly emerging human pathogen NESp, which are not protected against by the current pneumococcal vaccines.

Notes

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