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RE-EMERGENCE OF THE TYPE 1 PILUS AMONG *STREPTOCOCCUS PNEUMONIAE* ISOLATES IN MASSACHUSETTS, USA

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Abstract

Pneumococcal type 1 pilus proteins have been proposed as potential vaccine candidates. Following conjugate pneumococcal vaccination, the prevalence of the pneumococcal type 1 pilus declined dramatically, a decline associated with the elimination of vaccine-type (VT) strains. Here we show that between 2004 and 2007, there has been a significant increase in pilus prevalence, now exceeding rates from the pre-conjugate vaccine era. This increase is primarily due to non-VT strains. These emerging piliated non-VT strains are mostly novel clones, with some exceptions. The rise in pilus type 1 frequency across multiple distinct genetic backgrounds suggests that the pilus may confer an intrinsic advantage.

Keywords

S. pneumoniae pilus; PCV7; vaccine- and non-vaccine-types

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Introduction

Streptococcus pneumoniae is a common cause of infections including otitis media, pneumonia, meningitis, and bacteremia [1–2]. Human nasopharyngeal carriage is the source of transmission from person to person and serves as the first step in pathogenesis[3]. Nearly all children carry *S. pneumoniae* at some point in the first five years of life, and most carry many different strains over this period. Over 90 capsular types of *S. pneumoniae* are known, a subset of which are associated with carriage and disease in children and form the primary target of conjugate vaccine strategies.

Recently, pilus-like structures were identified in Gram-positive bacteria, including *S. pneumoniae*. The first type of pilus discovered in *S. pneumoniae* (now called a type 1 pilus following the discovery of a second type [4]) is encoded by a pathogenicity islet including genes for three structural proteins, *rrgABC*, three sortases, *srtBCD*, and a regulator, *rlrA*. It has been suggested that the type 1 pneumococcal pilus mediates host-bacterial interactions as an adhesin, a proinflammatory stimulus, and a virulence factor [5–6] and components of the pilus are being considered as vaccine candidates [7]. The pilus was present in approximately 25% of isolates in various populations prior to the widespread use of PCV7 [8–10], with no appreciable difference in frequency between carriage and disease isolates [8]. Furthermore, studies from different geographical locations have shown that the pilus is associated mainly with strains belonging to capsular types included in the 7-valent pneumococcal conjugate vaccine (PCV7) - vaccine-type (VT) strains [8,10].

In 2000, PCV7 was introduced for routine infant vaccination in the United States. Universal infant immunization with this vaccine was followed by a dramatic decrease in invasive pneumococcal infections, both among vaccinated age groups and among older children and adults, who had not received PCV7 [11–14]. This decrease was due to near total elimination of VT strains in the carried population, which was observed both in carriage and infection studies [15–17]. Among carriage isolates, there has been a complete replacement by serotypes not included in the vaccine (NVT), so that total carriage prevalence has not changed substantially, but these new types have been reported to cause significantly less disease than vaccine-types before the PCV7 era [1,11,13–14,18–21].

The initial decline in VT prevalence produced a decline in the prevalence of the type 1 pilus among invasive isolates in Massachusetts [8]. We hypothesized that this pattern would be reflected also in nasopharyngeal carriage isolates and that the decline would continue as VT pneumococci became even less common. We show here that the prevalence of piliated isolates did indeed decrease significantly among carriage isolates in the first few years after PCV7 introduction in Massachusetts. Surprisingly, however, in the years that followed (2004–7), type 1 piliated isolates re-emerged. In this study we report the changes in prevalence of piliated *S. pneumoniae* isolates in nasopharyngeal carriage isolates in Massachusetts from 2001 to 2007, and we update for 2007–8 the invasive isolate data previously reported by Basset et al. for 1998–2006 [8] to show that the invasive isolates showed a trend parallel to that of the carriage isolates. Finally, we attempt to elucidate possible reasons for the resurgence of the type 1 pilus in *S. pneumoniae* NP isolates.

Patients and Methods

Study population

1) Carriage study—Nasopharyngeal swabs were obtained by trained personnel from children less than 7 years old attending 8–16 pediatric practices in Massachusetts over 2–4 month periods during winter/spring of 2001, 2004 and 2007 as described [17], following parental informed consent. All study procedures were approved by the Harvard Pilgrim

Health Care institutional review board. Demographics of the study population, and serotype and drug-resistance characteristics of isolates were reported previously [17].

2) Invasive pneumococcal infection (IPD) study—All single patient pneumococcal isolates from blood cultures at Children's Hospital Boston (CHB) from January 2007 to December 2008 were serotyped and assessed for presence of the pilus as described below. The annual incidence of IPD and specifically of IPD caused by piliated isolates was compared to the recently published data from CHB in the 10 previous years[8].

Microbiologic Processing

Nasopharyngeal samples were processed within 24 hours of collection for *S. pneumoniae* growth as described previously[22]. Antibiotic susceptibility was determined by E-test (AB Biodisk, Solma, Sweden) using standard methods as detailed by Clinical and Laboratory Standards Institute (CLSI). CLSI susceptibility cutoffs were used to classify organisms as susceptible, intermediate or resistant to penicillin (sensitive≤0.06 μg/ml; intermediate=0.12–1.0 μg/ml; resistant≥2.0 μg/ml), ceftriaxone (sensitive≤0.5 μg/ml; intermediate=1.0 μg/ml; resistant≥2.0 μg/ml), azithromycin (sensitive≤0.5 μg/ml; intermediate=1.0 μg/ml; resistant≥2.0 μg/ml), and trimethoprim/sulfamethoxazole (sensitive≤0.5/9.5 μg/ml; intermediate=1/19–2/38 μg/ml; resistant≥4/76 μg/ml). Resistance to both penicillin and erythromycin was assessed and designated P+E. Serotypes were determined by the Quellung reaction. Serotypes included in the PCV7 were considered VT. Isolates whose serogroup was distinct from those in PCV7, as well as serotype 19A isolates, were classified for this study as non-vaccine type (NVT) [23]. Other isolates of PCV7 serogroups but not serotype (i.e.6A, 9N, 9V, and 23A/B) were considered vaccine-associated types (VAT). Multilocus sequence typing (MLST) was performed as described [24].

Detection of the presence of the type 1 pilus operon

Because *rrgC* (which encodes for one of the three structural pilus proteins) is highly conserved at the DNA level across strains [25] we defined an isolate as "pilus positive" if a PCR for the *rrgC* gene was positive. Briefly, to extract DNA, bacterial cells were boiled for 10 min in 1xPCR buffer (Takara Bio Inc., Shiga, Japan). For the PCR reaction 1 μL of the boilate s supernatant, 0.2 μL of *Taq* DNA polymerase with 1.25 μl of dNTP mix (Takara) and 0.1 μl of each primer were mixed in 1x PCR buffer in the reaction total volume of 25 μl. The reaction conditions consisted of 35 cycles of 94°C for 15s, 60°C for 15s and 72°C for 1 min, followed by 5 min at 72°C. The *rrgC* and *ply* specific primers were used in a single reaction to evaluate simultaneously the presence or absence of both genes: *rrgC* specific oligos c5 [5′-GCTCTGTGTTTTTCTCTTGTATGG-3′] and c3 [5′-ATCAATCCGTGGTCGCTTGTTATTTTTA-3′] and *ply* specific primers described by Whatmore et al. [26] were used. The PCR was repeated whenever *ply* specific product was not generated.

Statistical analysis

Data were analyzed using SAS software version 9.13 (SAS Institute, Cary, NC). Proportions of colonized children in the different years and type 1 piliated isolates and non-piliated isolates were calculated and compared using a Chi-square test. Stratifications that controlled for VT, antibiotic resistance or specific serotypes were carried out.

Results

Nasopharyngeal isolates from 2001, 2004 and 2007 were available; for these years, we obtained DNA samples from 122/190, 194/232 and 272/294 isolates respectively. In 2001, one year following PCV7 introduction, the prevalence of type 1 piliated isolates was 23.7%

(29/122) whereas in 2004, despite similar overall carriage rates[27], the prevalence of type 1 piliated isolates decreased to 14.9% (29/194, p=0.049). By 2007, the prevalence of type 1 piliated isolates had increased again to pre-PCV7 levels, representing 26.1% (71/272) of all *S. pneumoniae* isolates analyzed in that year (p=0.004, compared to 2004) (Figure 1).

The decline in the frequency of the type 1 pilus in nasopharyngeal isolates from 2001 to 2004 was largely attributable to the replacement of VT strains by NVT strains [27] in which the pilus was about 1/3 as common as in VT strains. However, between 2004 and 2007, the prevalence of the pilus increased, primarily due to an increase in its frequency among NVT strains; from 8.9% to 12.0%, and to 30.1% in 2001, 2004 and 2007 respectively, p=0.0002 (Figure 1).

The distribution of the type 1 pilus among isolates in our population was highly clonal, as previously reported [8–9]. Figures 2A and 2B show the distribution of the type 1 pilus among clones of two important non-vaccine serotypes. Consistent with this finding, we found that the emergence of piliated isolates among serotype 19A was due to the emergence of several piliated clones, within this serotype, mainly ST320 and ST695 (figure 2A). Similarly, the emergence of the pilus in serotype 35B strains was primarily due to a single piliated clone, ST558 (figure 2B).

To address the hypothesis that the return of the type 1 pilus might be due to its association with another, selectively advantageous trait such as drug resistance [17], we stratified the prevalence of piliated isolates by year and drug resistance pattern. Figure 3 shows that following a decline in type 1 pilus frequency among all strata of drug resistance from 2001–2004, there was an increase in frequency from 2004–2007 in all groups. Thus although the pilus was more common among isolates with reduced penicillin susceptibility and such isolates tended to be more common among replacing strains in 2007 [17], the rebound in prevalence cannot be fully explained by association with drug resistance.

If, on the other hand, the resurgence of the pilus resulted from a selective advantage of piliated strains in 2007, one would expect that the increased prevalence of these piliated strains would be observed repeatedly in different serotypes, just as it was for different antibiotic susceptibilities. Indeed, among the five most common serotypes found in 2007 (19A, 6A, 15B/C, 35B, and 11A) the prevalence of the pilus increased in all cases compared to 2004 (Figure 4).

We found that the prevalence of piliated strains was indeed inversely associated with age, in the age groups 7 months or older (trend p=0.002) (Figure 5), and was low among those under 7 months, as one would expect if maternal antibody conferred relative protection against colonization with piliated strains.

To determine whether the changes in prevalence of the pilus we observed for nasopharyngeal isolates were also observed for isolates from patients with IPD, we assessed single patient isolates detected in blood cultures. The number of cases in 2007 and 2008 was 13 and 16 cases respectively. As previously reported [8], the number of annual IPD cases in Children s Hospital Boston from 1998 to 2006 decreased gradually from ~35 cases to less than 7, similar to the trends observed in other USA centers[13,28–29]. While the prevalence of type 1 piliated strains among IPD cases was also reported to decrease from 1998 to 2006 (from 40% (30/77) to 10% (1/11) respectively, p=0.053) [8], in 2007 and 2008 the prevalence increased to 21% (6 of 29 cases, p=0.418). Although not statistically significant, these trends are similar to the prevalence trends of piliated strains among nasopharyngeal isolates.

Discussion

In this study we report the changing epidemiology of the type 1 pneumococcal pilus during a seven year period post-PCV7 implementation in the USA. Surprisingly, we found that after near elimination of VT strains among nasopharyngeal and IPD strains by 2007 and a parallel reduction in frequency of piliated isolates, a resurgence of piliated isolates followed, primarily among NVT pneumococci.

This resurgence might be due to an intrinsic selective advantage of the pilus, or to its association with genotypes carrying some other advantageous trait, such as antimicrobial resistance [30]. Indeed, the type 1 pilus was more common among penicillin-nonsusceptible strains in our study, and pen-NS serotypes were preferentially represented among replacing strains in this sample in 2007 [17]. However, this effect did not fully account for the increase in the type 1 pilus in 2007, since the increase was observed even within strata of drug resistance, and since the pilus increased from 2004 to 2007 without a corresponding increase in the prevalence of pen-I or pen-R strains. These findings indicate that antimicrobial selection pressure alone is unlikely to account for the increase in the frequency of piliated isolates.

Another hypothesis that may explain the resurgence of the pilus could be clonal expansion of a few successful NVT piliated clones (such as serotype 19A, ST 320). The type 1 pilus could then be growing in prevalence by “hitchhiking” with another unknown successful attribute of these expanding clones. While this possibility cannot be ruled out, we consider it relatively unlikely because the consistent increase in the pilus across multiple serotypes from 2004 to 2007 would require that this scenario have been repeated in many serotypes.

We have consistently observed an increase in type 1 pilus prevalence across multiple strata of serotype and drug sensitivity, and the preferential increase of piliated clones among the most prevalent serotypes. This is consistent with a selective advantage for piliated strains in 2007 relative to 2004. The fact that the frequency of piliated strains prior to PCV7 introduction in several populations was around 25%, and that it increased back to the same level by 2007, is more consistent with balancing selection (favoring the pilus only up to a certain frequency) than with directional selection, in which an advantage for piliated strains in all circumstances would be seen. In pathogens, a primary cause of frequency-dependent selection is host immunity, such that a determinant that aids the survival and transmission of an organism but is also a target of host immune responses, would be favored when rare (because few hosts would be immune) but disfavored when common [31]. If such a selective force were acting, one would expect the frequency of pilus to be highest in naïve hosts (those whose maternal antibody had waned and who had not yet acquired a robust immune response themselves) than in older infants or children, who would be likely to have acquired immunity. The finding that the prevalence of piliated strains was inversely associated with age is consistent with this hypothesis.

Thus, our data are consistent with the hypothesis that the pilus confers an intrinsic advantage for colonization, but that this advantage is partially counteracted in older hosts, perhaps because they are old enough to have developed immunity to the pilus.

Given the suggestion that this pilus may confer an advantage for colonization of at least some hosts, one might hypothesize that the rare prevalent NVT piliated strains in a non-vaccinated population could have predicted which NVT clones would expand after implementing universal vaccination in that population. However, our data cannot support this, since only five NVT piliated strains (of four clones) were prevalent in the early period (2001); ST558 (n=2), ST1946 (n=1), ST433 (n=1) and ST100 (n=1). Of these four sequence types, only ST558 has expanded, while the other three have disappeared by 2007.

Furthermore 13 other new NVT piliated clones have emerged. Another potential explanation could be that rare co-colonizing NVT strains, that were not identified were the source for expanding piliated NVT strains. The source of the expanding NVT piliated clones in 2007 is unclear from our data.

Our study is limited in its ability to fully describe time-trends, since only 3 time points were available. Since the first time point was 2001 (early post-vaccine era) we cannot determine the prevalence of piliated strains in the actual pre-vaccine era in this population or the prevalence of piliated NVT strains from that time. Thus, it may still be possible that the new NVT piliated clones have originated from pre-vaccine VT clones by capsular switching. Only a larger study that would examine many clones from the true pre-vaccine era (prior to 2000) could detect this.

In conclusion, we report here that after an initial decline in the prevalence of type 1 piliated strains in a population of children in Massachusetts, type 1 piliated strains have now returned at a frequency that is similar to that which was observed just around the time PCV7 was instituted[8]. The reasons for the reemergence of the pilus are not clear at this time. Additional studies in other geographical locations, both in pre-vaccinated and vaccinated population are required to determine whether the resurgence of the type 1 pilus can be observed in other settings and to elucidate the implications of this phenomenon worldwide.

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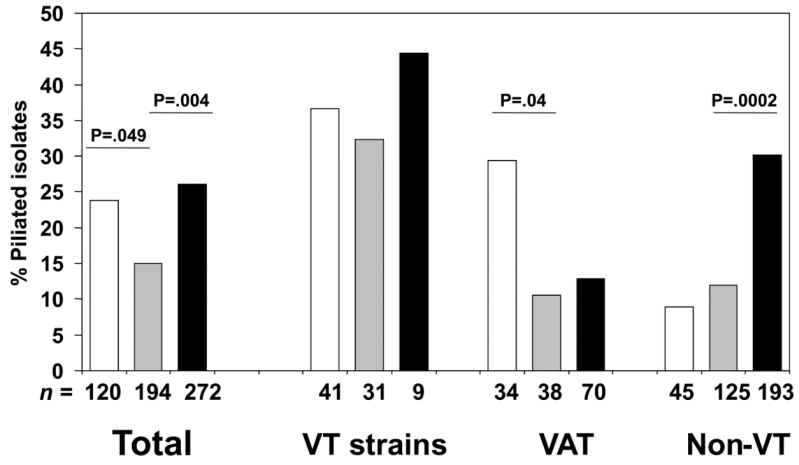

Fig. 1.

Prevalence of type 1 piliated isolates among all nasopharyngeal S. pneumoniae strains (Total) vaccine (VT), vaccine associated (VAT) and non-vaccine (non-VT) types and by year. White bars – 2001, grey bars – 2004, black bars – 2007. n is number among each group (VT, VAT or NVT) among all isolates in that year). Only significant p-values ($p<.05$) are shown.

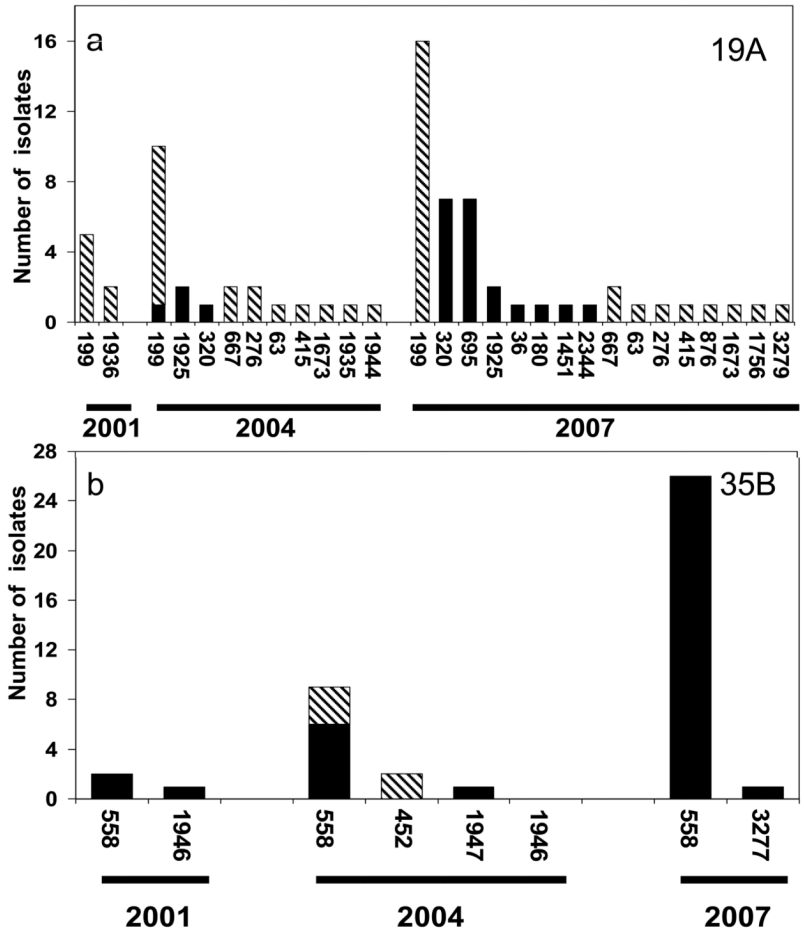

Fig 2.

Prevalence of type 1 pilus by ST for the 2 most common serotypes that emerged among nasopharyngeal isolates. Black – piliated isolates, Striped – non piliated isolates. a. Serotype 19A. b. Serotype 35B.

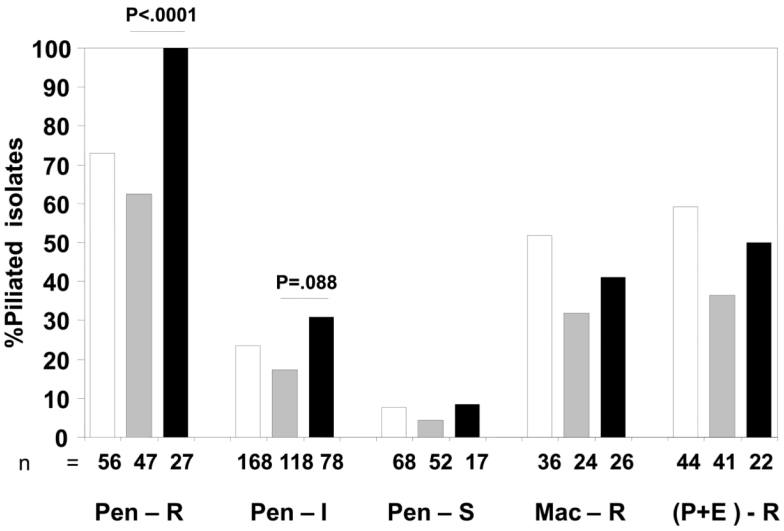

Fig. 3.

Prevalence of type 1 pilus among antibiotic resistance phenotypes of nasopharyngeal isolates. White bars – 2001, grey bars – 2004, black bars – 2007. *n* is number of isolates in each antibiotic group category (Pen-R, Pen-NS, Pen-S, Mac-R and (P+E) - R). % of strains in the category among all isolates in that year. P-values for 2004 to 2007 differences are shown where $p<0.1$.

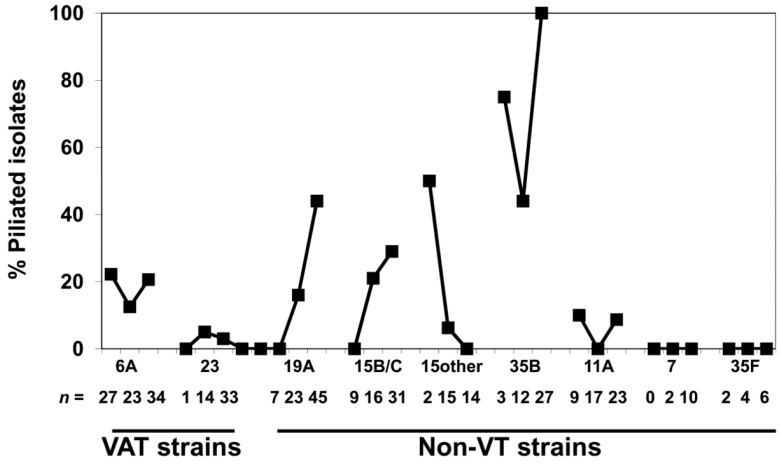

Fig. 4.

Proportion of type 1 piliated strains among VAT and non-VT nasopharyngeal strains. Only serotypes where there were more than 5 strains in at least one of the years are shown. For each serotype the 3 connected points represent % piliated strains in 2001–2004–2007.

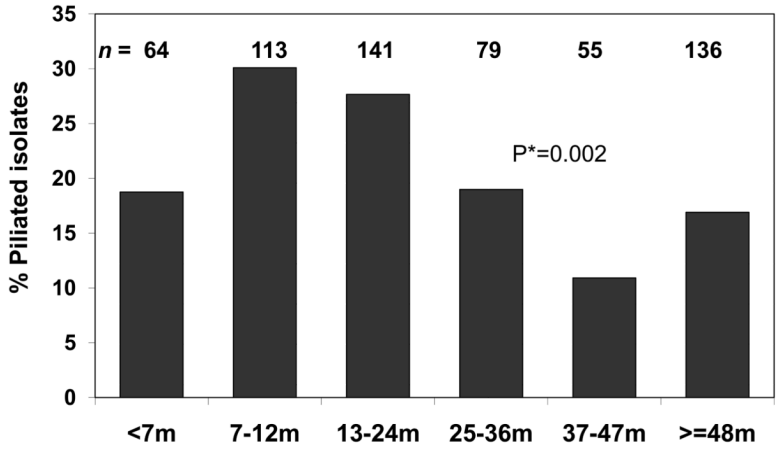

Fig. 5.

Prevalence of type 1 piliated isolates among nasopharyngeal carriage isolates by age. *Trend p calculated for age ≥ 7 months across categories using median age within category.