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ORIGINAL ARTICLE

Molecular characterization of *Streptococcus pneumoniae*, particularly serotype19A/ST320, which emerged in Krasnoyarsk, Russia

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ABSTRACT

***Streptococcus pneumoniae*, a common human pathogen, colonizes the nasopharynx and causes diseases including acute otitis media (AOM). Herein, pneumococcal serotype distributions in children before and after PCV7 vaccination and in patients with pneumococcal disease in Siberian Russia (Krasnoyarsk) are reported. Analyses included antimicrobial susceptibility testing, sequence typing (ST), pulsed field gel electrophoresis, virulence-related surface protein gene (VSG) typing with novel primers and structural analysis by scanning electron microscopy. In healthy children (HC) prior to administration of PCV7, drug-susceptible serotype23F/ST1500 was a major pneumococcal genotype. In the PCV7 trial, multidrug-resistant serotype19A/ST320 emerged in vaccinees after PCV7, exhibiting a PCV7-induced serotype replacement. Multidrug-resistant serotype19A/ST320 was evident in patients with AOM. Community-acquired pneumonia (CAP) isolates showed genetic similarities to the AOM (ST320) genotype, constituting a common non-invasive AOM–CAP group. In contrast, meningitis isolates were more divergent. Overall, 25 ST types were identified; five (20%) of which were Krasnoyarsk-native. Regarding VSGs, PI-1 (*rlrA*/*rrgB*), PI-2 (*pitA*/*B*), *psrP* and *cbpA* were present at 54.3%, 38.6%, 48.6%, and 95.7%, respectively, with two major VSG content types, PI-1^{-}/PI-2^{-}/*psrP*$^{+}$/*cbpA*$^{+}$ and PI-1^{+}/PI-2^{+}/*psrP*$^{-}$/*cbpA*$^{+}$, being found for HC and non-invasive diseases, respectively. A major clone of serotype19A/ST320 (PI-1^{+}/PI-2^{+}) produced the longest pneumococcal**

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List of Abbreviations: AOM, acute otitis media; CAP, community acquired pneumonia; CbpA, choline-binding protein A; CC, clonal complex; CLSI, Clinical and Laboratory Standards Institute; HC, healthy children, prior to the administration of PCV7; HIC, healthy PCV7-immunized children; MDR, multidrug resistance; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; PAP, pneumococcal adherent pili; PCV, pneumococcal conjugated vaccine; PFGE, pulsed-field gel electrophoresis; PsrP, pneumococcal serine-rich repeat protein; PSS, pneumococcal spike-like structure; PWS, pneumococcal wire (pilus) structure; SEM, electron microscopy; ST, sequence type; VSG, virulence-related surface protein gene.

wire (pilus) structures in colonies. ST1016 (PI-1$^-$/PI-2$^-$) in HC had HEp-2 cell-adherent pili. These results suggest that serotype19A/ST320 and related genotypes, with the VSG content type PI-1$^+$/PI-2$^+$/*psrP*$^-$/*cbpA*$^+$, emerged in vaccinees after PCV7 in Siberia, accompanying diseases in non-vaccinated children, and that some genotypes (serotypes19A/ST320 and 18/ST1016) produced novel pneumococcal structures, predicting their roles in colony formation and adherence.

Key words capsule/ST/surface protein gene type, PCV7 vaccination, pneumococcal wire (pilus) structure, *Streptococcus pneumoniae*.

Streptococcus pneumoniae, a leading bacterial human pathogen, has high morbidity and mortality rates (1). It colonizes the nasopharynx, particularly in children (1–3), nasopharyngeal colonization proceeding to development of pneumococcal diseases (1, 4–6). Invasive infections include bacteremia and meningitis; these are mostly reported in children aged <2 years (1). Mucosal/non-invasive infections include non-bacteremic pneumonia, AOM and sinusitis, which are less severe, but very common health issues (1, 6, 7). The most frequent *S. pneumoniae* infection is AOM, including patients in whom it is a complication of influenza (8).

The virulence factors of *S. pneumoniae* include capsular polysaccharides (9–12), which represent the serotypes of *S. pneumoniae*; more than 90 serotypes have been identified (5, 11, 13). A polysaccharide capsule plays a key role in nasopharyngeal colonization and immune evasion from phagocytosis or complement factor binding (10, 12, 13), and is the target of PCVs such as PCV7 and PCV13 (6, 11–14).

Regarding pneumococcal surface proteins (9–12), two types of pneumococcal pili, PI-1 and PI-2, have been identified. The major backbone subunit of PI-1 is the RrgB protein, the expression of which (gene, *rrgB*) is regulated by the *rlrA* gene (15–18). PI-1 is present only in certain pneumococcal CCs. The backbone protein of PI-2 is considered to be PitB (gene, *pitB*); the PI-2 islet carries a second pilus gene *pitA*, which has a premature stop codon (19, 20). Although PI-2 may be more widely distributed, it is weakly expressed (21).

The largest pneumococcal surface protein, PsrP, varies in size (22, 23). PsrP promotes pneumococcal adherence to nasopharyngeal epithelial cells and lung cells and contributes to robust formation of biofilms (9, 10, 22, 24–26). CbpA plays a role in adherence, colonization and immune evasion from complement attack and opsonophagocytosis (9, 10, 27).

S. pneumoniae forms biofilms during colonization of the nasopharynx and during diseases such as chronic otitis media and pneumonia (28, 29). Biofilms may endow pneumococci with reduced susceptibility to antimicrobial agents or resistance to immune recognition (10, 28). They may also contribute to colonization and persistence on the mucosa, but with altered virulence (24) or regulation (29).

S. pneumoniae serotypes/serogroups have been investigated in Russia (30, 31); three PCVs, PCV7, PCV10 and PCV13, have been registered for children. The molecular characteristics of *S. pneumoniae* have not yet been reported in Siberian Russia (Krasnoyarsk), which is located between the European and Far Eastern regions. We herein isolated and characterized *S. pneumoniae* in children before and after PCV7 vaccination and also in children with pneumococcal diseases in Krasnoyarsk. Because certain surface protein genes are highly variable, we designed PCR primers based on available pooled *S. pneumoniae* genome information. We also investigated pneumococcal surface ultrastructures using SEM.

MATERIALS AND METHODS

PCV7 vaccination, patients and ethics statement

None of the children in the present PCV7 trial had previously received *S. pneumoniae* vaccines. Furthermore, none of them received PCV7 before they presented with pneumococcal diseases. This study was approved by the Ethics Committee of Krasnoyarsk State Medical University (Protocol No. 2/2011). Informed consent was obtained from the parents or legal representatives of each child.

Typing of *S. pneumoniae*

Capsular typing was achieved by PCR, as previously described (32). STs were examined by PCR and sequencing, according to the MLST website (33) and CCs were analyzed using eBURST (34).

Susceptibility testing

The MICs of antimicrobial agents were measured by an agar dilution method using Mueller–Hinton agar (Difco, Sparks, MD, USA) supplemented with 5% sheep blood (Nippon Bio-Test Laboratories, Tokyo, Japan), as described previously (35, 36). MICs of antimicrobial agents (including penicillin) for *S. pneumoniae* ATCC49619 (reference strain) that were obtained by the agar dilution method were consistent with those described by the

CLSI (36). A disc method using antimicrobial agent discs (Becton Dickinson, Franklin Lakes, NJ, USA) was also employed (in initial susceptibility testing); oxacillin was used for penicillin in the disc method (36). Breakpoints for drug resistance were those described by CLSI (36). MDR was used when penicillin resistance (MIC $\geq$ 0.12 μg/mL for meningitis strains and MIC $\geq$ 8 μg/mL for non-meningitis strains [intermediate, MIC 4 μg/mL]) and resistance (or intermediate) to at least two more antimicrobial agents were included.

Clonal analysis of *S. pneumoniae*

In PFGE analysis, bacterial DNA was digested with *Sma*I and electrophoresed in 1.2% agarose, as described previously (37).

Surface protein gene analysis by PCR

The four VSGs, PI-1, PI-2, *psrP* and *cbpA*, were analyzed by PCR. The primers used for PCR are summarized in Table S1 (21, 38, 39) and are those reported by others plus those designed in the present study based on available pooled *S. pneumoniae* genome information (Fig. S1) (20, 21, 40, 41). The PCR conditions employed were initial denaturation at 95°C for 5 min, denaturation cycling at 95°C for 20 s, annealing at 52°C–55°C (depending on the Tm of primers used) for 30 s, extension at 68°C for 1 min (35 cycles), and a final extension at 68°C for 10 min, as described previously (39). PCR products were sequenced to confirm the target gene sequence.

Phylogenetic and homology analyses

A phylogenetic analysis was conducted in MEGA6 using the maximum likelihood method based on the Kimura 2-parameter model; the reliability of the tree was estimated with a bootstrap analysis by 1000 replicates (42, 43). A homology analysis was performed using BLAST software (44) and DNAMAN (45).

Analysis of *S. pneumoniae* ultrastructures

To investigate bacterial surface structures, *S. pneumoniae* was grown on trypticase soy agar supplemented with 5% sheep blood (Becton Dickinson, Tokyo) at 37°C for 12–18 hr. Blood agar-block pieces were fixed, dehydrated, critical-point dried, coated with gold-palladium and assessed using a SEM, as described previously (46). *S. pneumoniae* ATCC49619 was used as a control strain.

Adherence assay

Regarding adherence to HEp-2 cells (a human epithelial cell line originating from human laryngeal carcinoma), *S. pneumoniae* cells, grown on blood agar plates as above, were added to HEp-2 cells on plastic coverslips at 37°C for 2 hr, after which HEp-2 cell samples were assessed by SEM, as described previously (47). *S. pneumoniae* ATCC49619 was used as a control strain.

Statistical analysis

Data were statistically analyzed using Fisher's exact test. The level of significance was defined as $P < 0.05$.

RESULTS

First PCV7 trial in Krasnoyarsk

One hundred and eighty-three healthy children (aged 0–5 years; mean age, 3.7 years; 0.3% of the total population of approximately 70,000 children of corresponding ages in Krasnoyarsk), none of whom had any infectious diseases at the time of examination, were selected by pediatricians in 2011. Nasopharyngeal swabs were obtained from these healthy children prior to administration of PCV7 in 2011; these swabs yielded 93 *S. pneumoniae* strains. All 183 HC were then vaccinated with PCV7 in 2011. In 2013, nasopharyngeal specimens were obtained from 171 HIC; these swabs yielded 54 *S. pneumoniae* strains. The HIC did not receive antibiotics prior to the nasal swabs being obtained and *S. pneumoniae* isolated.

S. pneumoniae carriage rates in HC and HIC were 50.8% (93/183) and 31.6% (54/171), respectively, this being a significant decrease in carrier rate after vaccination ($p < 0.01$). *S. pneumoniae* carriage rates did not differ significantly between kindergarten-attending and non-attending children; most of the latter group of children had kindergarten- or school-attending siblings. Although vaccinees included residents of orphanages, no significant bias in carriage rates was observed between these orphans and other children because the orphans were in various orphanages located in different parts of Krasnoyarsk and were thus not members of a closed group.

PCV7 serotypes in HC, such as 23F and 19F, were controlled by administration of PCV7; the number of serotypes/serogroups changed from eight (23F, 19F, 15AF, 23A, 14, 6, 35AC/42 and 18) to five (19A, 15BC, 6, 35AC/42 and 11AD) (Fig. 1), but with prominent emergence of the non-PCV7 serotype 19A, confirming PCV7-related serotype replacement over the two-year period (48–50). There were no non-encapsulated strains.

Because the entire study group in Krasnoyarsk was disbanded in 2012, this trial yielded no accurate data concerning vaccine efficacy regarding disease prevention. Regarding HC, the serotype coverage rates of PCV7

and PCV13 were 62.5%; in the present study, we examined serogroup 6 only (not 6A, 6B or 6C).

S. pneumoniae strains were also isolated from patients with pneumococcal diseases in three hospitals in Krasnoyarsk. The most prevalent pathogen of AOM, a common disease in children, was *S. pneumoniae*, which accounted for 32.6% of bacterial infections (23.8% of all cases) in Krasnoyarsk; this is consistent with previous findings (51–54). The isolated strains included 12 strains from middle ear fluids of 12 patients with AOM (aged 8 months to 9 years; mean age, 2.0 years) in 2014 and 2016, four strains from aspirated sputum of four patients with CAP (aged 2 to 9 years; mean age, 6.2 years) in 2014–2016, and four strains from the blood of four patients with meningitis (aged 1 to 15 years; mean age, 8 years) in 2013–2015. Their serotypes/serogroups are summarized in Table 1. Regarding patients, the serotype coverage rates of PCV7 and PCV13 were 62.5% and 100%, respectively.

Molecular characterization of *S. pneumoniae*

Twenty-three HC and 27 HIC isolates were randomly selected from each serotype/serogroup and their molecular charcateristics examined; resultant data are summarized in Table 1. There were 11 ST types among the HC strains, the most prevalent being ST1500 (CC30)/serotype23F, which was drug-susceptible, whereas there were eight ST types among the HIC strains, the most prevalent being ST320(CC320)/serotype19A, which was MDR. MDR was observed only in CC320 and its related CC271 and CC236.

There were seven ST types among AOM strains ($n = 27$), the most prevalent being ST320/serotype19A, which was MDR (Table 1). CAP strains ($n = 4$) included CC271 and CC320; they were all MDR (Table 1). Meningitis strains ($n = 4$) were diverse in CCs; only CC320 was MDR (Table 1).

Regarding ST320/serotype19A strains, seven of the 10 HIC strains and two of the three AOM strains examined by PFGE (Fig. 2a) exhibited identical PFGE patterns (designated α1), two HIC strains showing only a one-band difference (PFGE pattern α2), indicating the same clone (ST320/19A$_{Kras}$). Regarding CAP strains (Fig. 2b), two strains (ST7915/serotype19A) showed the PFGE α1 pattern, whereas the remaining two (ST2323/serotype19F) showed a two-band difference (PFGE pattern α3) and belonged to the same clone (non-invasive group) as ST320/19A$_{Kras}$. In contrast, meningitis strains exhibited divergent PFGE patterns (Fig. 2b).

Regarding VSGs (Tables 1 and S2), when the 70 strains (Table 1) were examined, PI-1, PI-2, *psrP* and *cbpA* were found to be present at 54.3% (38/70), 38.6% (27/70), 48.6% (34/70), and 95.7% (67/70), respectively (Table 2). There were two major SVG content types, PI-1$^-$/PI-2$^-$/*psrP*$^+$/*cbpA*$^+$ and PI-1$^+$/PI-2$^+$/*psr*$^-$/*cbpA*$^+$; the latter correlated with a non-invasive AOM and CAP group (Table 2), mainly in association with CC320 and CC271 (Fig. 3). In contrast, the PI-1$^-$/PI-2$^-$/*psrP*$^+$/*cbpA*$^+$ type correlated with the colonization of HC (Table 2), mainly in association with CC30 and CC1025 (Fig. 3).

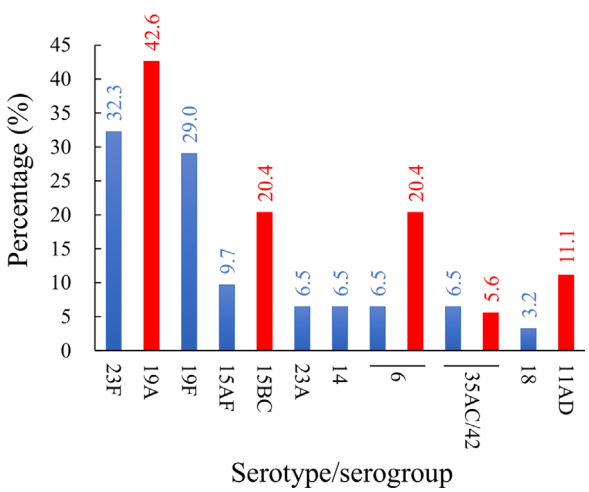

Fig. 1. Serotype/serogroup distribution in the first small-scale PCV7 trial in Krasnoyarsk, Siberian Russia. *S. pneumoniae* was isolated from the nasopharynxes of HC prior to administration of PCV7 in 2011 and then from HIC in 2013. Bars: blue, serotype/serogroup for HC; red, serotype/serogroup for HIC. Numbers above bars indicate percentages of each serotype/serogroup in the relevant group.

Ultrastructures of *S. pneumoniae*

S. pneumoniae cells grown on blood agar were analyzed by SEM (Fig. 4). When ST320/19A$_{Kras}$-α1 (Fig. 2a) was examined, very long PWS were found in the colonies (Fig. 4a, b). PWS were > 4 μm in length, gently curved, and had the appearance of whips or even peritrichous flagella; no short pili were observed. In the case of ST320/19A$_{Kras}$-α2 (Fig. 2a), *S. pneumoniae* cells in colonies only had shorter, straight, thin pili (Fig. 4c). *S. pneumoniae* ATCC49619, used as a control strain, showed no PWS or obvious pili.

When *S. pneumoniae* in HC (Table 1) was examined in the HEp-2 cell assay (Fig. 5), each single cell of ST1016/serogroup18 (PI-1$^-$/PI-2$^-$) was clearly piliated (Fig. 5a, arrow) and showed pili-mediated adherence (Fig. 5c); these pili (PAP) were often observed to form a ring at the position of cell septation sites (Fig. 5d; arrow). In contrast, no obvious pili-mediated adherence to HEp-2 cells was found for other

Table 1. Molecular characteristics of *S. pneumoniae* from children in Krasnoyarsk, Siberian Russia

Isolated from:	Isolation	Serotype or serogroup	ST (CC)	No. of strain	Resistance (resistant/total) i, intermediate	Virulence gene			
						PI-1	PI-2	*psrP*	*cbpA*
Healthy children prior to PCV administration ($n = 23$)	2011	23F	1500 (30)	8		−	−	+	+
			30 (30)	1		−	−	+	+
			8636†(‡)	1		−	−	+	-
		19F	236 (236)	1	P/Oi, E, C	+	−	−	+
			2323 (271)	1	P/Oi, Ei, T	+	+	−	+
			1203 (346)	1		−	−	−	+
		6	315 (315)	2	E,C,T	+	−	−	+
		35AC/42	1025 (1025)	2		−	−	+	+
		23A	8636† (‡)	2		−	−	+	+
		14	9250† (‡)	2		−	−	−	+
		18	1016 (102)	1		−	−	−	+
		15AF	6202 (6202)	1		−	−	−	+
Children, immunized with PCV7 ($n = 27$)	2013	19A	320 (320)	11	P/Oi(10/11), E, C(10/11),T(10/11)	+	+	−	+
		6	315 (315)	3	E,C,T	+	−	−	+
			9248† (‡)	3		+	+	−	+
			9247† (‡)	1		+	+	−	+
	15BC		1025 (1025)	3		−	−	+	+
			9249† (‡)	2		−	−	+	+
	11 AD		62 (62)	2		−	−	−	−
	35AC/42		1025 (1025)	2		−	−	−	+
Patients									
Acute otitis media	2014–2016	19A	320 (320)	5	P/O, E, C, Ti(1/5)	+	+	-	+
($n = 12$)		19F	1500 (30)	1		−	−	+	+
			271 (271)	1	P/O, E, C, T	+	+	−	+
			1464 (320)	2	P/O, E(1/2), C, T(1/2)	+	+	−	+
		23F	315 (315)	1	E,T	+	−	−	+
		9VA	156 (156)	1	P/O	+	+	−	+
		4	1637 (205)	1		+	−	−	+
Pneumonia	2014–2016	19F	2323 (271)	2	P/O, E,C	+	+	−	+
($n = 4$)		19A	7915 (320)	2	P/O, E,C	+	+	−	+
Meningitis	2013–2015	3	505 (180)	1		−	−	−	+
($n = 4$)		7AF	3544 (218)	1		−	+	−	+
		19F	9659 (320)	1	P/O, E,C,T	+	+	−	+
		6	5839 (‡)	1		−	−	−	+

C, clindamycin; E, erythromycin; O, oxacillin; P, penicillin; T, tetracycline.
PI-1$^+$: *rlrA*$^+$ and *rrgB*$^+$, *rlrA*$^+$, or *rrgB*+;
PI-1$^-$, *rlrA*$^-$ and *rrgB*$^-$.
†Novel ST found in Krasnoyarsk. ‡CC, not assigned.

S. pneumoniae in HC. *S. pneumoniae* belonging to ST320/19A $_{\text{Kras}}$-α1 and α2 (Fig. 2a) and *S. pneumoniae* ATCC49619, used as a control strain, also showed no adherence to HEp-2 cells (data not shown).

DISCUSSION

In Krasnoyarsk, Siberian Russia, *S. pneumoniae* found to be colonizing healthy children (HC and HIC) included five novel ST types (ST8636, ST9247, ST9248, ST9249 and ST9250), representing Krasnoyarsk (Siberia)-local characteristics. *S. pneumoniae* in HC, examined in 2011, was mostly drug-susceptible, the most prevalent type being ST1500(CC30)/serotype23F with the SVG content type PI-1$^-$/PI-2$^-$/*psrP*$^+$/*cbpA*$^+$. The genotype *psrP*$^+$ in HC is consistent with previous reports that PsrP promotes nasopharyngeal pneumococcal colonization (25, 55). Although the serotypes were divergent, ST1500 was also a minor constituent of isolates from patients with AOM (between 2014 and 2016). CC30 is

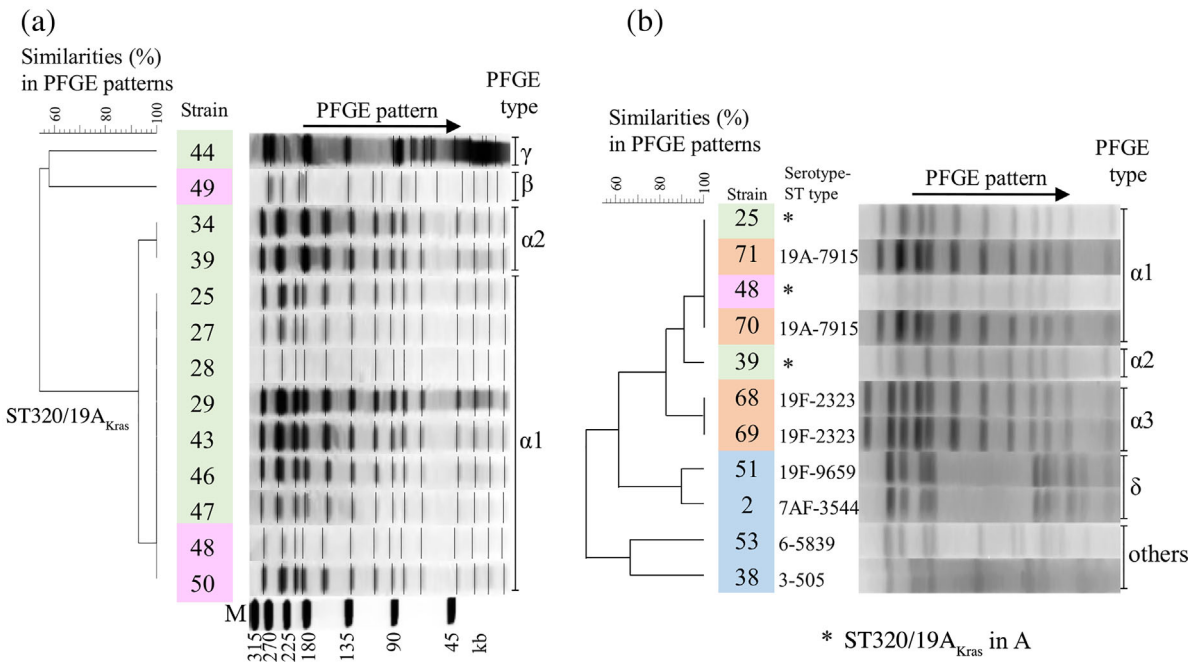

Fig. 2. PFGE analysis of *S. pneumoniae* from Krasnoyarsk, Siberian Russia. (a) Comparison of ST320/serotype19A *S. pneumoniae* from patients with AOM and healthy HIC. (b) Comparison of *S. pneumoniae* from patients with pneumonia and meningitis with representative PFGE types of ST320/serotype19A *S. pneumoniae*. Color in strain column: pink, AOM isolates; green, HIC isolates; orange, pneumonia isolates; blue, meningitis isolates. M, molecular size standard (lambda ladder).

not a global CC and, to the best of our knowledge, there is currently no information available on its genome. A few of the *S. pneumoniae* colonizing HC were MDR; ST236(CC236)/serotype19F and ST2323(CC271)/serotype19F were found to be related to MDR ST320 (CC320)/serotype19A (Figs. S2, S3).

The first small-scale PCV7 trial in Krasnoyarsk resulted in serotype replacement with global, MDR ST320/serotype19A (Fig. S3), which is consistent with previous findings (48–50, 56–58). Nasopharyngeal colonization precedes any type of pneumococcal disease, including AOM. PCV7 primarily increases serotype 19A carriage and, as a consequence, increases the rate of 19A-related pneumococcal disease. In the present study, because HIC did not receive antibiotics prior to isolation of *S. pneumoniae*, the underlying mechanism or source

Table 2. Distribution of virulence-related surface protein genes (VSGs) and VSG content types in *S. pneumoniae* isolates

S. pneumoniae-isolation group (number of children)	PCR-positive percent of the gene/region (positive/total)			
	PI-1	PI-2	*psrP*	*cbpA*
Healthy children (HC), prior to the PCV7 administration (n = 23)	17% (4/23)	4% (1/23)	87% (20/23)	96% (22/23)
Healthy PCV7-immunized children (HIC) (n = 27)	67% (18/27)	41% (11/27)	44% (12/27)	93% (25/27)
Disease, total (n = 20)	80% (16/20)*1	75% (15/20)*1	10% (2/20)*1	100% (20/20)
acute otitis media (n = 12)	92% (11/12)*2	75% (9/12)*2	17% (2/12)*2	100% (12/12)
Pneumonia (n = 4)	4/4	4/4	0/4	4/4
acute otitis media+ pneumonia (n = 16)	94% (15/16)*3	81% (13/16)*3	13% (2/16)*3	100% (16/16)
Meningitis (n = 4)	1/4	2/4	0/4	4/4
P value, vs. that of HC	*1, $P < 0.01$ *2, $P < 0.01$ *3, $P < 0.01$	*1, $P < 0.01$ *2, $P < 0.01$ *3, $P < 0.01$	*1, $P < 0.01$ *2, $P < 0.01$ *3, $P < 0.01$	

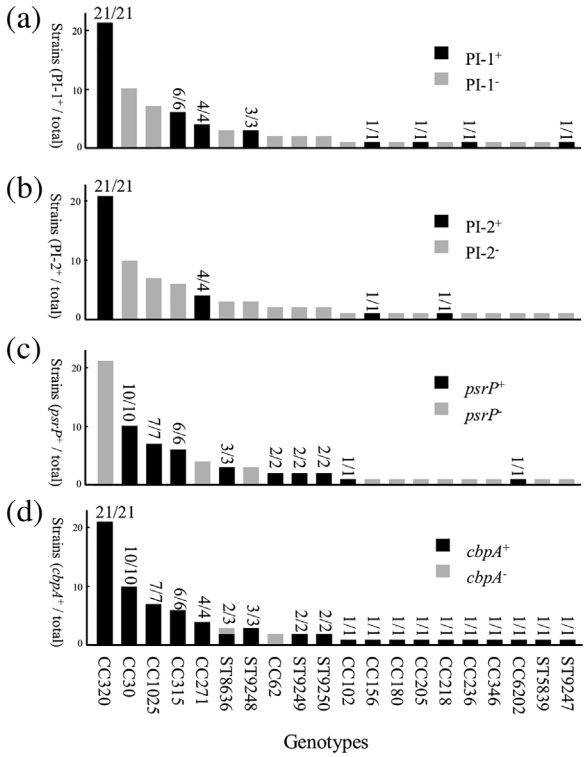

Fig. 3. Distribution of virulence-related surface protein genes (regions), PI-1, PI-2, psrP and cbpA, for each *S. pneumoniae* genotype. (a) PI-1; (b) PI-2; (c) *psrP*; (d) *cbpA*. Histograms show the numbers of gene-positive/total strains, stratified by the CC or ST; positive-strain numbers are shown by dark-colored boxes. CCs were not assigned for the ST types shown.

of emergence in HIC of ST320/serotype19A, which appeared to have a high fitness cost from MDR (59), currently remains unknown. ST320/serotype19A may have superior fitness to the "empty" nasopharyngeal niche in HIC. Moreover, in the present study we did not examine carriage in healthy children who had not been vaccinated with PCV7.

Of the four VSGs, PI-1, PI-2, *psrP* and *cbpA* are located at a region (named *rlrA* pathogenicity islet or PI-1 islet) flanked by IS*1167* (21, 60), the PI-2 islet (20, 21), a pathogenicity island (22, 26, 61), and a chromosomal core (in some cases, a region flanked by IS) (41, 62), respectively. Certain VSGs had highly variable sequences, confirming previous findings (41). Therefore, we improved the PCR primers and also attempted to use multiple primers for each PCR target. As a result, the low yields (for example, 55.7%-positive) in PCR using previously reported primers increased to high yields (for example, 94.3%) in the present study, reaching the level predicted on the basis of pooled genome sequences previously reported. However, a limitation of the present study was that we only analyzed four VSG targets and the disease groups studied, particularly CAP and meningitis, were small. Results of PFGE analysis and VSG content typing strongly suggest that AOM and CAP isolates are a common non-invasive group. The results of the present study also indicate that the surface proteins PitA and PitB are protected from attacks by host immunity

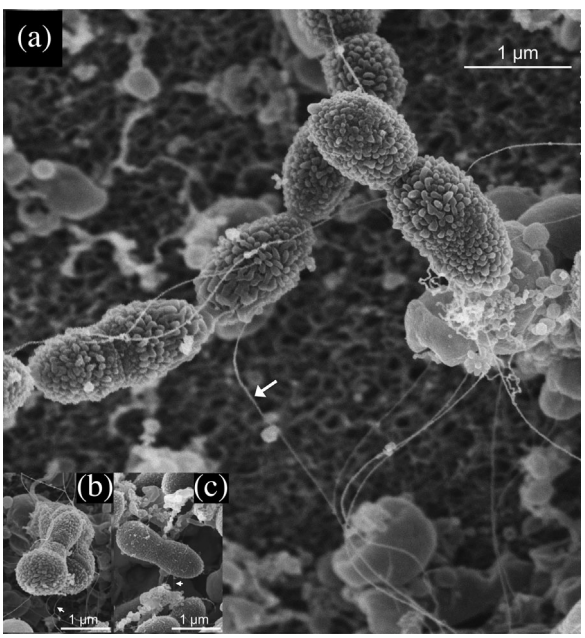

Fig. 4. Scanning electron micrographs showing cell surface structures of ST320/serotype19A *S. pneumoniae* grown on blood agar plates. (a, b) PFGE α1 type (ST320/19A$_{Kras}$-α1, Fig. 2a) of ST320/serotype19A was examined; *S. pneumoniae* has gently curved PWS (arrow). (c) PFGE α2 type (ST320/19A$_{Kras}$-α2, Fig. 2a) was examined; *S. pneumoniae* have short, thin, straight pili (arrow).

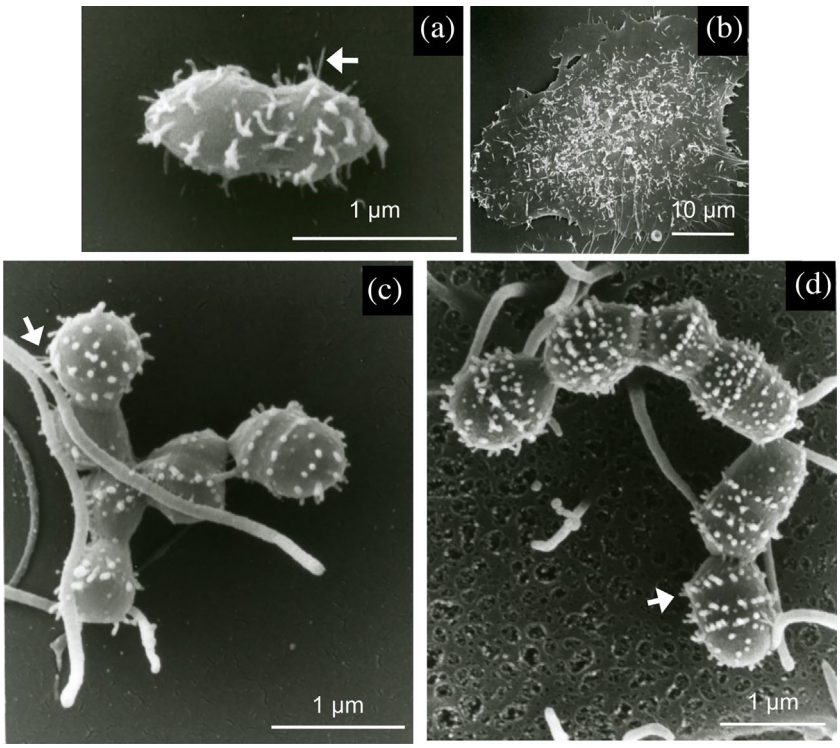

Fig. 5. Scanning electron micrographs showing cell surface structures of ST1016/serogroup18 *S. pneumoniae* examined in a HEp-2 cell adherence assay. (a) Single ST1016/serogroup18 cell with pili (arrow) on a plastic coverslip. (b) HEp-2 cell. (c, d) Adherence of ST1016/serogroup18 cells to HEp-2 cells with PAP (arrow).

because, even though *rrgB*, *psrP* and *cbpA* were highly variable, *pitA* and *pitB* were conserved.

In previous studies, we found that it was possible to demonstrate the unique surface structures of bacterial pathogens in colonies (at high cell densities) using SEM (46, 63, 64). In the present study, we found that the major epidemiological clone (PFGE α1 type) of ST320/serotype19A, associated with AOM, formed novel PWS in colonies; the PWS were unusually long, curved, and did not have the morphological appearance of pili, instead resembling whips or peritrichial flagella. AOM-associated ST320/serotype19A was PI-1^+/PI-2^+. The molecular and genetic features of PWS are currently being investigated. Type IV pili, very long pilus structures, have been investigated in gram-negative bacteria (65). We also noted heavy biofilm formation and PSS in colonies of AOM-associated ST320/serotype19A (Figs. S4, S5). At high cell densities in colonies, these structures may adhere together to make rigid, non-invasive clinical foci on the mucosa, thus facilitating successful colonization and infection.

We also found that, in HC, ST1016/serogroup18 formed HEp-2 cell-adherent pili. Given that ST1016/serogroup18 was PI-1^-/PI-2^-, PAP represent the third most frequent adherent pili in *S. pneumoniae* that play roles in colonization of HC.

In conclusion, we, for the first time, here report changes in pneumococcal serotype distribution in a cohort of children after PCV7 vaccination and in a small group of patients with pneumococcal disease in Krasnoyarsk, Siberian Russia. We found Krasnoyarsk (Siberia)-local clones. The most prevalent *S. pneumoniae* in the nasopharyngeal niche of HC was drug-susceptible ST1500/serotype23F with the VSG content type PI-1^-/PI-2^-/*psrP*$^+$/*cbpA*$^+$, suggesting that PsrP plays an important role in nasopharyngeal colonization. PCV7 increased the carriage in HIC of MDR serotype 19A/ST320, with PI-1^+/PI-2^+/*psrP*$^-$/*cbpA*$^+$ and, as a consequence, increased the rate of 19A/ST320-related non-invasive pneumococcal disease, including AOM, in non-vaccinated children. The major epidemiological clone of ST320/serotype19A (PI-1^+/PI-2^+) formed novel PWS in colonies, most likely for rigid colonization and immune evasion in the middle ear. ST1016/serogroup18 (PI-1^-/PI-2^-) in HC formed novel HEp-2 cell-adherent pili, possibly for nasopharyngeal colonization and was the third most frequent pneumococcal pili.

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DISCLOSURE

The authors declare that they have no conflicts of interest.

REFERENCES

1. World Health Organization. (2012) Pneumococcal vaccines WHO position paper–2012. *Wkly Epidemiol Rec* **87**: 129–44.
2. Roche A., Heath P.T., Sharland M., Strachan D., Breathnach A., Haigh J., Young Y. (2007) Prevalence of nasopharyngeal carriage of pneumococcus in preschool children attending day care in London. *Arch Dis Child* **92**: 1073–6.
3. Verstrheim D.F., Hùiby E.A., Aaberge I.S., Caugant D.A. (2008) Phenotypic and genotypic characterization of *Streptococcus pneumoniae* strains colonizing children attending day-care centers in Norway. *J Clin Microbiol* **46**: 2508–18.
4. de Lencastre H., Tomasz A. (2002) From ecological reservoir to disease: The nasopharynx, day-care centres and drug-resistant clones of *Streptococcus pneumoniae*. *J Antimicrob Chemother* **50 Suppl S2**: 75–81.
5. Devine V.T., Jefferies J.M., Clarke S.C., Faust S.N. (2015) Nasopharyngeal bacterial carriage in the conjugate vaccine era with a focus on pneumococci. *J Immunol Res* **2015**: 394368.
6. Centers for Disease Control and Prevention. *Pneumococcal disease*. 8 September 2015. Available from: http://www.cdc.gov/vaccines/pubs/pinkbook/pneumo.html
7. Weil-Olivier C., van der Linden M., de Schutter I., Dagan R., Mantovani L. (2012) Prevention of pneumococcal diseases in the post-seven valent vaccine era: A European perspective. *BMC Infect Dis* **12**: 207.
8. Greene C.J., Marks L.R., Hu J.C., Reddinger R., Mandell L., Roche-Hakansson H., King-Lyons N.D., Connell T.D., Hakansson A.P. (2016) Novel strategy to protect against influenza virus-induced pneumococcal disease without interfering with commensal colonization. *Infect Immun* **84**: 1693–703.
9. Jedrzejas M.J. (2001) Pneumococcal virulence factors: Structure and function. *Microbiol Mol Biol Rev* **65**: 187–207.
10. Steel H.C., Cockeran R., Anderson R., Feldman C. (2013) Overview of community-acquired pneumonia and the role of inflammatory mechanisms in the immunopathogenesis of severe pneumococcal disease. *Mediators Inflamm* **2013**: 490346.
11. Geno K.A., Gilbert G.L., Song J.Y., Skovsted I.C., Klugman K.P., Jones C., Konraden H.B., Nahm M.H. (2015) Pneumococcal capsules and their types: Past, present, and future. *Clin Microbiol Rev* **28**: 871–99.
12. Berical A.C., Harris D., Dela Cruz C.S., Possick J.D. (2016) Pneumococcal vaccination strategies: An update and perspective. *Ann Am Thorac Soc* **13**: 933–44.
13. Rodrigo C., Lim W.S. (2014) The relevance of pneumococcal serotypes. *Curr Infect Dis Rep* **16**: 403.
14. Alderson M.R. (2016) Status of research and development of pediatric vaccines for *Streptococcus pneumoniae*. *Vaccine* **34**: 2959–61.
15. Barocchi M.A., Ries J., Zogaj X., Hemsley C., Albiger B., Kanth A., Dahlberg S., Fernebro J., Moschioni M., Masignani V., Hultenby K., Taddei A.R., Beiter K., Wartha F., von Euler A., Covacci A., Holden D.W., Normark S., Rappuoli R., Henriques-Normark B. (2006) A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci USA* **103**: 2857–62.
16. Hilleringmann M., Giusti F., Baudner B.C., Masignani V., Covacci A., Rappuoli R., Barocchi M.A., Ferlenghi I. (2008) Pneumococcal pili are composed of protofilaments exposing adhesive clusters of Rrg A. *PLoS Pathog* **4**: e1000026.
17. Basset A., Turner K.H., Boush E., Sayeed S., Dove S.L., Malley R. (2012) An epigenetic switch mediates bistable expression of the type 1 pilus genes in *Streptococcus pneumoniae*. *J Bacteriol* **194**: 1088–91.
18. Figueira M., Moschioni M., De Angelis G., Barocchi M., Sabharwal V., Masignani V., Pelton S.I. (2014) Variation of pneumococcal pilus-1 expression results in vaccine escape during experimental otitis media [EOM]. *PLoS ONE* **9**: e83798.
19. Shaik M.M., Lombardi C., Marango Trindade D., Fenel D., Schoehn G., Di Guilmi A.M., Dessen A. (2015) A structural snapshot of type II pilus formation in *Streptococcus pneumoniae*. *J Biol Chem* **290**: 22581–92.
20. Zähner D., Gandhi A.R., Yi H., Stephens D.S. (2011) Mitis group streptococci express variable pilus islet 2 pili. *PLoS ONE* **6**: e25124.
21. Bagnoli F., Moschioni M., Donati C., Dimitrovska V., Ferlenghi I., Facciotti C., Muzzi A., Giusti F., Emolo C., Sinisi A., Hilleringmann M., Pansegrau W., Censini S., Rappuoli R., Covacci A., Masignani V., Barocci M.A. (2008) A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol* **190**: 5480–92.
22. Shivshankar P., Sanchez C., Rose L.F., Orihuela C.J. (2009) The *Streptococcus pneumoniae* adhesin PsrP binds to keratin 10 on lung cells. *Mol Microbiol* **73**: 663–79.
23. Schulte T., Löfling J., Mikaelsson C., Kikhney A., Hentrich K., Diamante A., Ebel C., Normark S., Svergun D., Henriques-Normark B., Achour A. (2014) The basic keratin 10-binding domain of the virulence-associated pneumococcal serine-rich protein PsrP adopts a novel MSCRAMM fold. *Open Biol* **4**: 130090.
24. Sanchez C.J., Kumar N., Lizcano A., Shivshankar P., Dunning Hotopp J.C., Jorgensen J.H., Tettelin H., Orihuela C.J. (2011) *Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. *PLoS ONE* **6**: e28738.
25. Dockrell D.H., Whyte M.K., Mitchell T.J. (2012) Pneumococcal pneumonia: Mechanisms of infection and resolution. *Chest* **142**: 482–91.
26. Sanchez C.J., Shivshankar P., Stol K., Trakhtenbroit S., Sullam P.M., Sauer K., Hermans P.W., Orihuela C.J. (2010) The pneumococcal serine-rich repeat protein is an intra-species

bacterial adhesin that promotes bacterial aggregation *in vivo* and in biofilms. *PLoS Pathog* **6**: e1001044.
27. Chen A., Mann B., Gao G., Heath R., King J., Maissoneuve J., Alderson M., Tate A., Hollingshead S.K., Tweten R.K. Briles D.E., Tuomanen E.I., Paton J.C. (2015) Multivalent pneumococcal protein vaccines comprising pneumolysoid with epitopes/fragments of CbpA and/or PspA elicit strong and broad protection. *Clin Vaccine Immunol* **22**: 1079–89.
28. Hall-Stoodley L., Hu F.Z., Gieseke A., Nistico L., Nguyen D., Hayes J., Forbes M., Greenberg D.P., Dice B., Burrows A., Wackym P.A., Stoodley P., Post J.C., Ehrlich G.D., Kerschner J.E. (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* **296**: 202–11.
29. Chao Y., Marks L.R., Pettigrew M.M., Hakansson A.P. (2015) Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. *Front Cell Infect Microbiol* **4**: 194.
30. Rudakova A.V., Baranov A.A., Lobzin Yu V., Briko N.I., Namazova-Baranova L.S., Tatochenko V.K., Kharit S.M., Sidorenko S.V., Koroleva I.S., Kozlov R.S., Mayanskii N.A., Kostinov M.P., Snegova N.F. (2014) [Pharmacoeconomic assessment of 13-valent pneumococcal conjugate vaccine in immunization of children in Russian Federation]. *Voprosy sovremennoi pediatrii* **13**: 51–9. (In Russian)
31. Mayanskiy N., Alyabieva N., Ponomarenko O., Lazareva A., Katosova L., Ivanenko A., Kulichenko T., Namazova-Baranova L., Baranov A. (2014) Serotypes and antibiotic resistance of non-invasive *Streptococcus pneumoniae* circulating in pediatric hospitals in Moscow, Russia. *Int J Infect Dis* **20**: 58–62.
32. Centers for Disease Control and Infection. *Streptococcus Laboratory*. [Cited November, 2012] Available from URL: http://www.cdc.gov/streplab/pcr.html
33. Department of Infectious Disease Epidemiology, Imperial College, London. *Multi Locus Sequence Typing*. [Cited November, 2012] Available from URL: http://www.mlst.net/
34. Department of Infectious Disease Epidemiology, Imperial College, London. *eBURSTv3* [Cited November, 2012] Available from URL: http://eburst.mlst.net/
35. Takano M., Ozaki K., Nitahara Y., Higuchi W., Takano T., Nishiyama A., Yamamoto T. (2009) *Streptococcus pneumoniae* and *Haemophilus influenzae* at the initial stage of influenza. *Pediatr Int* **51**: 687–95.
36. CLSI. (2015) *CLSI Document M100-S25* Wayne, PA: Clinical and Laboratory Standards Institute.
37. McEllistrem M.C., Stout J.E., Harrison L.H. (2000) Simplified protocol for pulsed-field gel electrophoresis analysis of *Streptococcus pneumoniae*. *J Clin Microbiol* **38**: 351–3.
38. Moschioni M., Donati C., Muzzi A., Masignani V., Censini S., Hanage W.P., Bishop C.J., Reis J.N., Normark S., Henriques-Normark B., Covacci A., Rappuoli R., Barocchi M.A. (2008) Streptococcus pneumoniae contains 3 rlrA pilus variants that are clonally related. *J Infect Dis* **197**: 888–96.
39. Sakai F., Talekar S.J., Lanata C.F., Grijalva C.G., Klugman K.P., Vidal J.E., RESPIRA PERU Group. (2013) Expression of Streptococcus pneumoniae virulence-related genes in the nasopharynx of healthy children. *PLoS ONE* **8**: e67147.
40. Muñoz-Almagro C., Selva L., Sanchez C.J., Esteva C., de Sevilla M.F., Pallares R., Orihuela C.J. (2010) PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia and is strongly associated with clonal type. *Clin Vaccine Immunol* **17**: 1672–8.
41. Donati C., Hiller N.L., Tettelin H., Muzzi A., Croucher N.J., Angiuoli S.V., Oggioni M., Dunning Hotopp J.C., Hu F.Z., Riley D.R., Covacci A., Mitchell T.J., Bentley S.D., Kilian M., Ehrlich G.D., Rappuoli R., Moxon E.R., Masignani V. (2010) Structure and dynamics of the pan-genome of Streptococcus pneumoniae and closely related species. *Genome Biol* **11**: R107.
42. Hall B.G. (2013) Building phylogenetic trees from molecular data with MEGA. *Mol Biol Evol* **30**: 1229–35.
43. Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013) MEGA6: Molecular evolutionary genetic analysis version 6.0. *Mol Biol Evol* **30**: 2725–9.
44. US National Library of Medicine and National Center for Biotechnology Information. *BLAST*. [Cited June, 2016] Available from URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi
45. Lynnon Corporation. *DNAMAN – Bioinformatics Solutions*. [Cited December, 2016] Available from URL: http://www.lynnon.com/index.html
46. Yamamoto T., Takano T., Yabe S., Higuchi W., Iwao Y., Isobe H., Ozaki K., Takano M., Reva I., Nishiyama A. (2012) Super-sticky familial infections caused by Panton-Valentine leucocidin-positive ST22 community-acquired methicillin-resistant *Staphylococcus aureus* in Japan. *J Infect Chemother* **18**: 187–98.
47. Yamamoto T., Kaneko M., Changchawalit S., Serichantalergs O., Ijuin S., Echeverria P. (1994) Actin accumulation associated with clustered and localized adherence in *Escherichia coli* isolated from patients with diarrhea. *Infect Immun* **62**: 2917–29.
48. Richter S.S., Heilmann K.P., Dohrn C.L., Riahi F., Diekema D.J., Doern G.V. (2013) Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999–2011. *Emerg Infect Dis* **19**: 1074–83.
49. Croucher N.J., Chewapreecha C., Hanage W.P., Harris S.R., McGee L., van der Linden M., Song G.H., Ko K.S., de Lencastre H., Turner P., Bentley S.D. (2014) Evidence for soft selective sweeps in the evolution of pneumococcal multidrug resistance and vaccine escape. *Genome Biol Evol* **6**: 1589–602.
50. Tóthpál A., Laub K., Kardos S., Tirczka T., Kocsis A., van der Linden M., Dobay O. (2015) Epidemiological analysis of pneumococcal serotype 19A in healthy children following PCV7 vaccination. *Epidemiol Infect* **144**: 1563–73.
51. Rovers M.M., Schilder A.G.M., Zielhuis G.A., Rosenfeld R.M. (2004) Otitis media. *Lancet* **363**: 465–73.
52. Coker T.R., Chan L.S., Newberry S.J., Limbos M.A., Suttorp M.J., Shekelle P.G., Takata G.S. (2010) Diagnosis, microbial epidemiology, and antibiotic treatment of acute otitis media in children: A systematic review. *JAMA* **304**: 2161–9.
53. Monasta L., Ronfani L., Marchetti F., Montico M., Vecchi Brumatti L., Bavcar A., Grasso D., Barbiero C., Tamburlini G. (2012) Burden of disease caused by otitis media: Systematic review and global estimates. *PLoS ONE* **7**: e36226.
54. Liebertal A.S., Carroll A.E., Chonmaitree T., Ganiats T.G., Hoberman A., Jackon M.A., Joffe M.D., Miller D.T., Rosenfeld R.M., Sevilla X.D., Schwartz R.H., Thomas P.A., Tunkel D.E. (2013) The diagnosis and management of acute otitis media. *Pediatrics* **131**: e964–99.
55. Schulte T., Mikaelsson C., Beaussart A., Kikhney A., Deshmukh M., Wolniak S., Pathak A., Ebel C., Löfling J., Fogolari F., Henriques-Normark B., Dufrêne Y.F., Svergun D., Nygren P.Å., Achour A. (2016) The BR domain of PsrP interacts with extracellular DNA to promote bacterial aggregation; structural insights into pneumococcal biofilm formation. *Sci Rep* **6**: 32371.
56. Klugman K.P. (2011) Contribution of vaccines to our understanding of pneumococcal disease. *Philos Trans R Soc Lond B Biol Sci* **366**: 2790–8.

57. Cohen R., Varon E., Doit C., Schlemmer C., Romain O., Thollot F., Béchet S., Bonacorsi S., Levy C. (2015) A 13-year survey of pneumococcal nasopharyngeal carriage in children with acute otitis media following PCV7 and PCV13 implementation. *Vaccine* **33**: 5118–26.
58. Kim L., McGee L., Tomczyk S., Beall B. (2016) Biological and epidemiological features of antibiotic-resistant *Streptococcus pneumoniae* in pre- and post-conjugate vaccine eras: A United States Perspective. *Clin Microbiol Rev* **29**: 525–52.
59. Vogwill T., MacLean R.C. (2015) The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl* **8**: 284–95.
60. Telford J.L., Barocchi M.A., Margarit I., Rappuoli R., Grandi G. (2006) Pili in gram-positive pathogens. *Nat Rev Microbiol* **4**: 509–19.
61. Obert C., Sublett J., Kaushal D., Hinojosa E., Barton T., Tuomanen E.I., Orihuela C.J. (2006) Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. *Infect Immun* **74**: 4766–77.
62. Hall B.G. (2013) Building phylogenetic trees from molecular data with MEGA. *Mol Biol Evol* **30**: 1229–35.
63. Yamamoto T., Takano T., Higuchi W., Hung W.C., Reva I., Yabe S., Iwao Y., Khokhlova O. (2013) Unique features of the motility and structures in the flagellate polar region of *Campylobacter jejuni* and other species: An electron microscopic study. *Microbiol Immunol* **57**: 83–90.
64. Lin Y.T., Tsai J.C., Yamamoto T., Chen H.J., Hung W.C., Hsuen P.R., Teng L.J. (2016) Emergence of a small colony variant of vancomycin-intermediate *Staphylococcus aureus* in a patient with septic arthritis during long-term treatment with daptomycin. *J Antimicrom Chemother* **71**: 1807–14.
65. Craig L., Pique M.E., Tainer J.A. (2004) Type IV pilus structure and bacterial pathogenecity. *Nat Rev Microbiol* **2**: 363–78.

SUPPORTING INFORMATION

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Fig S1. Structures of virulence-related surface protein genes, *cbpA*, *rrgB*, *pitA*, *pitB* and *psrP*, and the new PCR primer design. (a) *cbpA*. (b) *rrgB*. (c1) *pitA*. (c2) *pitB*. (d1 to d3) *psrP*. (①) Phylogenetic tree analysis. (②) Nucleotide sequence comparison. (③) Amino acid sequence comparison. (①) Strain types are shown as ST (CC)/serotype. Strains marked in yellow were analyzed for conserved or divergent regions at the nucleotide sequence (②) and amino acid sequence (③) levels. In (②) and (③), homologous regions are shaded. Abbreviations in (③): Cna_B, Cna protein B-type domain; DUF11, domain of unknown function (gram-positive pilin backbone subunit 2); GramPos_pilinBB (DUF11 super family); GramPos_pilinD1, gram-positive pilin subunit D1; GramPos_pilinD3, gram-positive pilin backbone subunit 3; LPXTG, LPXTG motif for cell wall anchoring (in b-③, d1-③, d2-③, d3-③); RICH, Rich In CHarged residues (in a-③); VTPTG, the VTPTG motif (in c2-③); VWA_2, von Willebrand factor type A domain (in c1-③). New PCR primers were designed to target the conserved region of each gene. The PCR primers indicated in (②) are described in Table S1. Regarding *cbpA* (a), the *cbpA* sequence varied greatly. Although the primer cbpA-F/cbpA-R was designed within a conserved region, when the 70 strains (Table 1) were examined, the primer JVS73L/JVS74R sequences targeted a less homologous region (a-②, ③), yielding 94.3%-positive and 55.7%-positive results, respectively. Regarding *rrgB* (b), *rrgB* encodes the major backbone subunit, RrgB, of PI-1. *rrgB* exhibits a limited number of clusters in a phylogenetic analysis (b-①), but with a highly divergent sequence for each cluster (b-②, ③). Although the primer rrgB-F/rrgB-R was designed within a conserved region, the primer JVS69L/JVS70R targeted a highly variable region, yielding 52.9%-positive and 40.0%-positive results, respectively, for the 70 strains (Table 1). Regarding *pitA* (c1) and *pitB* (c2), *pitA* and *pitB* are located in PI-2 islet; the *pitB* product, PitB, is the backbone protein of PitB pilus. A second pilus gene *pitA* has a premature stop codon (20, 21). *pitA* of ST320/19A strains from AOM in the present study also had a premature stop codon. *pitA* and *pitB* also exhibit a limited number of clusters in the phylogenetic analysis (c1-①, c2-①). However, each gene sequence is highly conserved (c1-②, ③; c2-②, ③). The *pitA* primers, P06 for/P06 rev, pitA-F/pitA-R, and pitB-F/pitB-R, all yielded 38.6% for the 70 strains (Table 1). Regarding *psrP* (d1) to (d3), *psrP* sequences were classified into two major groups, about 14 kpb *psrP* (d1) and about 4 kb *psrP* (d2), designated as *psrP(S)*. In (d3), *psrP* and *psrP(S)* were compared, revealing a marked difference in the size of the serine-rich repeat region on the C-terminal side. *psrP* (about 14 kpb in size) is highly variable according to phylogenetic analysis (d1-①). The primer JVS77L/JVS78R targets the conserved region on the 3′-terminal (C-terminal) side, whereas the primer psrP-F/psrP-R targets that on the 5′-terminal (N-terminal) side (d1-②, ③). The target sequence of the primer JVS77L/JVS78R is present in 8/10 genome/*psrP* sequences searched and absent in 2/5 genome/*psrP(S)* sequences. However, that of the primer psrP-F/psrP-R is present in all sequences searched, including *psrP(S)*. The primers psrP-F/psrP-R and JVS77L/JVS78R yielded 48.6% and 28.6%-positive results, respectively, for the 70 strains (Table 1). All CC320 (and its related CC236 and CC271) strains in the present study were negative for *psrP* in PCR, which is consistent with the negative results obtained in our search of reported CC320 genome sequences; similar negative findings have previously been reported (40, 41).
Fig S2. Genetic relationships of CC236/CC271/CC320 lineages of *S. pneumoniae* in Krasnoyarsk, Siberian

Russia. Of the 70 strains (Table 1) examined, 26 (37.1%, 26/70) belonged to the complex CC271/CC236/CC320 lineages, including ST types (236, 271, 320, 1464, 2323, 7915 and 9659). (a) ST types and their allelic profiles of the CC271/CC236/CC320 strains of *S. pneumoniae* are shown. Among the seven genes (*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl*) used for ST typing (allelic profiling), *ddl* varied the most, resulting in four types: *ddl*1, *ddl*26, *ddl*106, and *ddl*610. *aroE* and *xpt* are occasionally responsible for additional variations. (b) The nucleotide sequences of the *ddl* genes were analyzed for phylogenetic diversity. (c) The genetic relationship of each ST type is shown for serotype 19F or serotype 19A.

Fig S3. Isolation and distribution of CC236/CC271/CC320 lineages of *S. pneumoniae* in Krasnoyarsk, Siberian Russia. The CC236/CC271/CC320 lineages, including ST types (236, 271, 320, 1464, 2323, 7915 and 9659) that were analyzed as shown in Fig. S2, were isolated in a PCV7 trial before and after PCV7 (a, b) and from non-immunized pediatric patients with pneumococcal diseases (a, c). MDR ST320/serotype19A (marked in green) was isolated from HIC in 2013 and from patients with AOM in 2014–2016. MDR ST232/serotype19F (marked in orange) was isolated from HC prior to administration of PCV7 in 2011 and from patients with pneumonia in 2014–2016.

Fig S4. Scanning electron micrographs showing a colony of ST320/serotype19A *S. pneumoniae* on blood agar plates. The PFGE α1 type (ST320/19A$_{Kras}$-α1, Fig. 2a) was examined. The central region of the colony (a) had very heavy biofilms (b and c). (c) Arrow, *S. pneumoniae* in heavy biofilms. *S. pneumoniae* at the colony center (near the edge) has PWS. (b) On the left, an arrow indicates *S. pneumoniae* with PWS. In contrast, *S. pneumoniae* cells located outside of colonies (near the edge) had no detectable PWS (right arrow in b).

Fig S5. Scanning electron electronmicrograph showing PSS. *S. pneumoniae*, serotype19A/ST320 PFGE α1 type (19A/ST320$_{Kras}$-α1, Fig. 2a), was grown on blood agar. Arrow, PSS; arrowhead, PWS.

Table S1. Primers used for the PCR assay

Table S2. Yields in PCR for each primer