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Antimicrobial resistance, penicillin-binding protein sequences, and pilus islet carriage in relation to clonal evolution of *Streptococcus pneumoniae* serotype 19A in Russia, 2002–2013

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SUMMARY

Clonal changes of serotype 19A pneumococci have been appreciated in conjunction with growing prevalence of this serotype after implementation of the seven-valent pneumococcal conjugate vaccine (PCV7). In the present study, we characterized serotype 19A pneumococci collected in Russia within a decade preceding the implementation of PCV vaccination and described their clonal evolution. We retrospectively analyzed non-invasive serotype 19A isolates collected in 2002–2013. All isolates were subjected to multilocus sequence typing, antimicrobial susceptibility testing, determination of macrolide resistance genotype, molecular detection of pilus islet (PI) carriage, sequencing of penicillin-binding protein (PBP) genes. A total of 49 serotype 19A isolates represented 25 sequence types, of which 14 were newly described. The majority of isolates were distributed among clonal complex (CC) 663 (28%), CC230 (25%), CC156, and CC320 (14% each). CC663 and CC156 dominated in 2003, but were replaced by CC230 and CC320 later on; CC320 was only evident starting 2010. All isolates of CC663 and CC156 carried PI1; CC320 possessed both PI1 and PI2. The overall rate of altered amino acids in penicillin-nonsusceptible isolates was 13·9%, 7·2%, and 8·7% for PBP1a, PBP2b, and PBP2x, respectively. Our findings demonstrate that the clonal structure of serotype 19A pneumococci may evolve without PCV pressure.

Key words: Antimicrobial resistance, multilocus sequence typing, penicillin-binding protein, pneumococcus, serotype 19A.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is an important human pathogen that may cause a wide range of infections including severe invasive disease such as bacteremia and meningitis. Introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in the USA in 2000 and, later, in other countries has strongly reduced the incidence of PCV7 vaccine serotype invasive pneumococcal disease (IPD) there [1–3]. At the same time, a growing rate of non-PCV7 vaccine serotypes, primarily serotype 19A, has been documented among circulating pneumococci in the post-PCV7 period [4, 5]. In the USA, the incidence of serotype 19A-related IPD has increased from 0·7 to 2·5–2·6 cases per 100 000 population between 1999 and 2008 [6]. According to data from

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Germany, the proportion of serotype 19A isolates among IPD pneumococci has increased up to 15% in 2010–2011 from a basal range of 1·7–4·2% observed in the period between 1997 and 2006 [7]. A similar trend was observed in other European countries after implementation of universal PCV7 vaccination [8–10]. These increases were attributed to the PCV-mediated serotype replacement phenomenon, i.e. the proliferation of pre-existing minor non-PCV7 serotype pneumococcal populations under vaccine pressure [11]. However, in some territories, for instance, in Israel and South Korea, a rise of serotype 19A was observed in the pre-PCV7 period [12–14] suggesting that, in addition to serotype, other bacterial factors might be relevant for the expansion.

The proliferation of serotype 19A pneumococci displays clonality. Pneumococcal clones, or clonal complexes (CCs), are readily distinguishable by multilocus sequence typing (MLST) [15, 16]. They demonstrate a variable distribution over time and territory as well as diversity in terms of virulence, antimicrobial resistance, and overall fitness [17]. Reportedly, only a few clones have contributed to the expansion of serotype 19A including CC199, CC230, CC320, CC695, and CC994 [6–8, 10, 18–23]. Most of these clones have reduced antimicrobial susceptibility revealing a multidrug resistant (MDR) phenotype with high level of resistance to penicillin, which is related to alterations of penicillin-binding proteins (PBPs) [24]. The clone of sequence type (ST) 320, the major, globally emerged serotype 19A clone, and CC199 (ST416) have been shown to carry pili that may provide an additional selective advantage [10, 23, 25].

Data on pneumococcal molecular epidemiology in Russia are limited. A recent report from our group documented the presence of serotype 19A CC320 in Russia before implementation of PCV vaccination in the National immunization program in 2014 [26]. In the present study, we retrospectively examined a set of non-invasive serotype 19A pneumococci collected in Russia during 2002–2013. We describe clonal diversity changing over this time period in conjunction with antimicrobial susceptibility, pilus prevalence, and PBP sequence alterations.

METHODS

Isolate collection

This retrospective study included serotype 19A pneumococcal isolates obtained from children from different parts of Russia during 2002–2013. One part of this collection was selected from a set of non-invasive isolates recovered by the Institute of Antimicrobial Chemotherapy, Smolensk, Russia, in 2002–2008 [27]. Another part of the collection comprised of serotype 19A pneumococci discovered during a multicenter study conducted in Moscow in 2010–2013 at the Scientific Centre of Children's Health [28]. The isolates were obtained from respiratory tract specimens, including nasopharyngeal swabs, middle ear fluid, sputum, and bronchoalveolar lavage, i.e. were non-invasive. No invasive serotype 19A isolates were recovered in these studies.

Serotyping and antimicrobial susceptibility testing

Laboratory examination of the isolates was performed at the Scientific Centre of Children's Health. All isolates underwent repeated serotyping by Quellung reaction using Staten Serum Institut antisera (Copenhagen, Denmark). Antimicrobial susceptibility for penicillin (PEN), amoxicillin (AMX), ceftriaxone (CTX), and erythromycin (ERY) was examined by the E-test (bioMerieux, France); the remaining antimicrobials including clindamycin (CLI), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), and tetracycline (TET) were tested by the disk diffusion method (Bio-Rad, Hercules, CA). Minimum inhibitory concentration (MIC) category and zone diameter breakpoint interpretations were based on updated EUCAST-2013 standards. Pneumococcal isolates with PEN MICs >0·06 mg/l and >2 mg/l were regarded as PEN-nonsusceptible and PEN-resistant, respectively. A PCR was used to detect the *erm(B)* and *mef* determinants in ERY-resistant pneumococci, as described previously [28]. An MDR phenotype was defined as nonsusceptibility to three or more classes of antibiotics.

MLST procedure

MLST analysis was performed according to the *S. pneumoniae* MLST protocol [15, 16]. Alleles and STs were assigned using the software at the pneumococcal web page (http://pubmlst.org/spneumoniae). Analysis of the STs and assignment to CC was performed against all STs found in the online database using the eBURST program. The STs that shared at least five of seven allelic variants composed a CC. Trace files for six new MLST alleles (*recP*, $n = 2$; *dll*, $n = 4$) and eight new MLST profiles were submitted to the MLST database for designations.

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Detection of the pilus islets 1 and 2 (PI1 and PI2)

The presence of PI1 was detected by PCR amplification of an internal fragment of the *rlrA* gene using primers *RLRA*-F (5′-TCTGATAGATGAGACGCTGT TG-3′) and *RLRA*-R (5′-CTCCGCTTCTTTCTA CTACAAG-3′), as described elsewhere [29]. For detection of PI2, primers for PCR amplification of conserved regions within the PI2 were modified from Bagnoli *et al*. [30] as follows: *PI2*-F (5′-CGTGGG TATCAGGTGTCCTATG-3′) and *PI2*-R (5′-TGCA GTGAATAGCTTTTTAAAGAA-3′).

Amplification, sequencing, and genetic analyses of *pbp1a, pbp2b, and pbp2x genes*

Fragments of *pbp1a, pbp2b,* and *pbp2x* including the coding regions of the penicillin-binding domains and adjacent sequences were amplified from genomic DNA and sequenced using the Sanger method, as described elsewhere [31]. Amplified fragments corresponded to the following amino acid positions: PBP1a, positions 341–582; PBP2b, positions 375–652; PBP2x, positions 337–661. Deduced amino acid sequences were compared with the corresponding sequences of the reference strain *S. pneumoniae* R6; pneumococcal clones of Taiwan^{19F}-14 (ST236), Spain^{9V}-3 (ST156), and MDR19A (ST320). We used the same GenBank accession numbers, as described earlier [32]. The relatedness of discovered amino acid sequences was depicted using neighbor-joining trees constructed with Molecular Evolutionary Genetics Analysis (MEGA) software [32].

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 20·0 (IBM Corp, Armonk, NY). Contingency table analysis for comparing proportions was done by the χ^2 test, or by means of the *z*-test, where appropriate. The tests were considered statistically significant at $P < 0{\cdot}05$.

RESULTS

Description of the serotype 19A pneumococcal collection

This study included 49 serotype 19A pneumococcal non-invasive isolates obtained from children (median age, 2·76 years; IQR (interquartile range) 2·59 years). Eighteen isolates were collected in 2003 in European Russia (cities of Moscow, Saint Petersburg, and Smolensk); nine isolates were obtained in Asian Russia in 2002–2008 (cities of Anadyr, Irkutsk, Novokuznetsk, and Yekaterinburg); 22 isolates were discovered in Moscow in 2010–2013 (Supplementary Table S1). The overall rate of 19A pneumococci was 6·4% (30 of 468; 27 isolates were available for further analyses) and 2·2% (22 of 1002) for the 2002–2008 and 2010–2013 collections, respectively ($Z = 3{\cdot}7$, $P < 0{\cdot}001$). The isolates were recovered from the nasopharynx (67%), middle ear fluid (18%), and lower respiratory tract specimens (sputum and bronchoalveolar lavage fluid; 15%).

Clonal evolution and antimicrobial resistance

The clonal lineage and ST diversity of serotype 19A pneumococci from time and geographic perspective is presented in Table 1 and Fig. 1. Overall, 25 STs were discovered, including 14 newly described STs, that belonged to 6 major clonal lineages; three STs were singletons. The distribution of clonal lineages over the study period was significantly different ($\chi^2 = 29{\cdot}60$, $P < 0{\cdot}001$). The European-2003 isolates were represented by only two clonal lineages, CC663 ($n = 12$) and CC156 ($n = 6$). In contrast, among isolates recovered in 2010–2013, these clones had a minor prevalence being largely replaced by CC230 and CC320, which collectively comprised 73% (16 of 22) of the 2010–2013 isolates. Five of nine strains from Asian Russia were singletons; three isolates belonged to CC230.

The most abundant clone, the clone of ST663 ($n =$ 14, 28% of the entire collection), was represented by four related STs (Table 1). CC663 predominated in European Russia in 2003 (67%); in 2010–2013, only two CC663 isolates were recovered. The majority of CC663 representatives (79%) had an MDR phenotype with high β-lactam MICs (Tables 2 and 3). All CTX-resistant isolates ($n = 4$; MIC > 2 mg/l) belonged to this CC (Table 3). Moreover, the isolates of ST10434 ($n = 5$) had an extremely drug-resistant phenotype being nonsusceptible to five out of six antibiotic groups tested, including PEN, ERY, CLI, CHL, and TET (Supplementary Table S1). Among the examined antibiotics, the ST10434 isolates were only susceptible to SXT.

The second most prevalent clonal lineage was CC230 ($n = 12$, 25% of the entire collection) that included eight STs (Table 1). The majority of CC230 isolates were recovered in Moscow during 2010–2013 contributing to 41% for this part of the collection;

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Table 1. *Clonal complexes (CCs) and sequence types (STs) of serotype 19A pneumococci isolated in Russia, 2002–2013*

CC	ST	No. of isolates (% of all isolates)	No. of isolates (% of isolates in the corresponding column)			Comment*
			European Russia		Asian Russia	
			2003	2010–2013	2002–2008	
663		**14 (28%)**	**12 (67%)**	**2 (9%)**†	**0**	**Colombia^{23F}-26 (ST338)**
	663		5	1		
	10 434		4	1		DLV of ST663
	10 435		2	0		DLV of ST663
	10 515		1	0		SLV of ST663
230		**12 (25%)**	**0**	**9 (41%)**†	**3 (33%)**	**Denmark^{14}–32 (ST230)**
	230			2	1	
	276			1	0	
	1611			1	0	
	2013			1	0	
	5369			0	1	
	5539			1	0	
	10 431			2	1	DLV of ST230
	10 432			1	0	TLV of ST230
156		**7 (14%)**	**6 (33%)**	**1 (5%)**†	**0**	**Spain^{9V}-3 (ST156)**
	143		0	1		
	10 437		4	0		DLV of ST156
	10 438		1	0		SLV of ST 10 437
	10 514		1	0		SLV of ST 10 437
320		**7 (14%)**	**0**	**7 (32%)**†	**0**	**Taiwan^{19F}-14 (ST236)**
	320			3		
	9656			4		SLV of ST320
Singletons		**5 (10%)**	**0**	**0**	**5 (56%)**‡	
	10 433			0	1	New allele combination
	10 436			0	1	New *dll* sequence
	10 512			0	3	New allele combination
Miscellaneous		**4 (10%)**	**0**	**3 (13%)**	**1 (11%)**	
	63			1	0	Sweden^{15A}-25 (ST63)
	5954			0	1	Group of two (ST5954, ST10430)
	10 430			1	0	SLV of ST5954
	10 511			1	0	Netherlands^{3}–31 (ST180)
Total		**49 (100%)**	**18 (100%)**	**22 (100%)**	**9 (100%)**	

* PMEN clones and their identifier STs (in brackets) related to the corresponding CC are indicated in bold typeface. For newly described STs, the relatedness to the corresponding clone identifier ST is shown.

† Significant differences between the proportions of 2003 and 2010–2013 ($P < 0{\cdot}05$).

‡ Significant differences between the proportions of 2002–2008 and 2003, 2010–2013 ($P < 0{\cdot}05$).

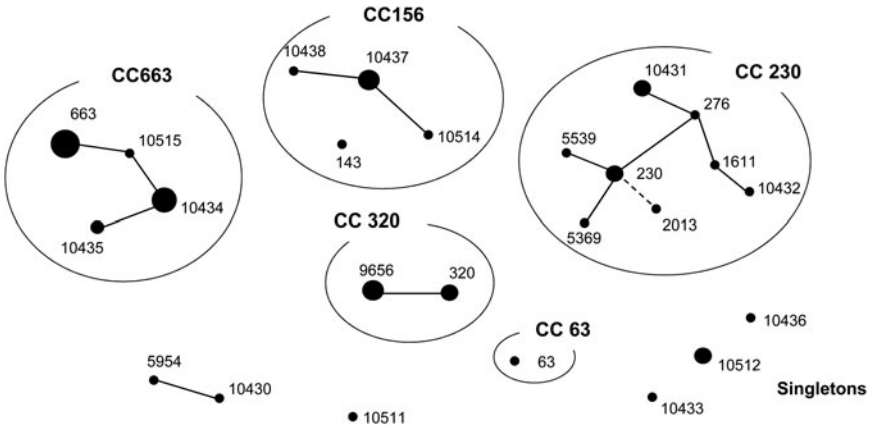

Fig. 1. Population snapshot of serotype 19A pneumococci recovered in Russia, 2002–2013, using eBURST analysis. Solid lines indicate SLVs; a dashed line indicates DLV. Circle size is proportional to the number of isolates.

three isolates was found in Asian Russia. CC230 was characterized by moderate-to-low β-lactam MICs being largely susceptible to CLI, CHL, and TET, but resistant to SXT (Tables 2 and 3). An MDR phenotype was evident in five (42%) of the CC230 isolates that were ERY-resistant (Supplementary Table S1).

CC156 was represented by seven serotype 19A pneumococci of four STs accounting for 14% of the entire collection. Six out of seven isolates were collected in European Russia in 2003 composing a subclone of three closely related new STs including ST10437, ST10438, and ST10514 (Table 1). These 19A pneumococci possessed a remarkable phenotype being fully resistant to AMX with MICs 4–8 mg/l that exceeded PEN MICs (Table 3, Supplementary Table S1). In addition, these isolates were resistant to SXT, but susceptible to ERY, CLI, CHL, and TET.

CC320 (seven isolates, 14% of the entire collection) was evident exclusively in 2010–2013 having a 32% prevalence (Table 1). It was represented by two STs: ST320 ($n = 3$) and ST9656 ($n = 4$; a new SLV of ST320 at the *recP* locus). All CC320 isolates displayed an MDR phenotype having high PEN and AMX MICs (range 1·0–4·0 mg/l), resistance to ERY and SXT (Tables 2 and 3). All but one isolate had higher MICs for AMX than for PEN; all isolates were susceptible to CHL (Supplementary Table S1).

Nine isolates represented various clonal lineages or unrelated STs. All six PEN-susceptible serotype 19A pneumococci belonged to this group of isolates (Tables 2 and 3). None of the PEN-susceptible isolates had an MDR phenotype. One singleton (ST10436) had high PEN and AMX MICs (2 mg/l) (Table 3).

In the European isolates, the overall nonsusceptibility rate to tested β-lactams was similar in 2003 and 2010–2013; however, the proportion of fully PEN-resistant isolates (MIC > 2 mg/l) declined from 28% (5 of 18) to 5% (1 of 22) during this period ($P = 0{\cdot}041$). Although the prevalence of ERY-resistant serotype 19A pneumococci remained unchanged (67–68%), the proportion of ERY-resistant isolates carrying both *erm*(B) and *mef* increased from 17% (2 of 12) in 2003 to 60% (9 of 15) in 2010–2013 ($P = 0{\cdot}023$). The resistance rate to CHL significantly declined over time from 33% (6 of 18) to 5% (1 of 22) ($P = 0{\cdot}017$). In contrast, the prevalence of SXT-resistant isolates increased from 44% (8 of 18) to 82% (18 of 22) ($P = 0{\cdot}014$) during the study period.

The prevalence of PI carriage in relation to clonal distribution and antimicrobial resistance

In total, 29 isolates (59%) showed the presence of PI1, of which seven isolates carried PI2 in addition; 20 isolates (41%) were PI-negative (Table 2, Supplementary Table S1). The prevalence of PI1 and PI2 had a clear clonal pattern. CC663 and CC156 pneumococci showed the presence of PI1 but not PI2, whereas the CC320 isolates harbored both PIs (Table 2). Among the remaining pneumococci, no isolates carried a PI except for the singleton of ST10436 which was PI1-positive. The rate of MDR phenotypes was significantly higher among PI-positive isolates (Table 4). The vast majority of piliated isolates were nonsusceptible to β-lactam antibiotics, whereas all PI-negative isolates were susceptible to AMX and CTX and had low-to-moderate PEN MICs (Table 4, Supplementary

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Table 2. *Antimicrobial resistance, macrolide resistance genotype, and pilus islet (PI) carriage within individual clonal groups of serotype 19A pneumococci*

CC (No. of isolates)	No. (%) of isolates											
	Nonsusceptible to the corresponding antimicrobial									Macrolide resistance genotype*	PI1†	PI2
	PEN	AMX	CTX	ERY	CLI	SXT	CHL	TET	MDR			
CC663 (14)	14 (100%)	13 (93%)	11 (79%)	14 (100%)	9 (64%)	3 (21%)	7 (50%)	6 (43%)	11 (79%)	*erm(B)*/*mef*, 3 (21%); *erm (B)*, 6 (43%); *mef*, 3 (21%); no *erm*(B)/*mef*, 2 (14%)	14 (100%)	0
CC230 (12)	12 (100%)	0	0	5 (42%)	2 (17%)	12 (100%)	1 (8%)	2 (17%)	5 (42%)	*erm*(B)/*mef*, 2 (40%); *mef*, 3 (60%)	0	0
CC320 (7)	7 (100%)	7 (100%)	7 (100%)	7 (100%)	6 (86%)	7 (100%)	0	4 (57%)	7 (100%)	*erm*(B)/*mef*, 6 (86%); *mef*, 1 (14%)	7 (100%)	7 (100%)
CC156 (7)	7 (100%)	7 (100%)	4 (57%)	1 (14%)	1 (14%)	6 (86%)	0	1 (14%)	1 (14%)	*erm*(B), 1	7 (100%)	0
CC63 (1)	0	0	0	1	1	0	0	1	1	*erm*(B), 1	0	0
Singleton, 10 433 (1)	1	0	0	1	1	0	0	0	1	*erm*(B), 1	0	0
Singleton, 10 436 (1)	1	1	1	0	0	1	0	0	0	No ERY-R	1	0
PEN-S‡ (6): CC180, ST10511 (1); singleton, ST10512 (3); group of two STs, ST5954 (1), ST10430 (1)	0	0	0	0	0	4 (67%)	0	1 (17%)	0	No ERY-R	0	0
Overall (49)	43 (88%)	28 (57%)	23 (47%)	29 (59%)	20 (41%)	33 (67%)	8 (16%)	13 (27%)	25 (51%)	*erm*(B)/*mef*, 11 (38%); *erm* (B), 9 (31%); *mef*, 7 (24%); no *erm*(B)/*mef*, 2 (7%)	29 (59%)	7 (14%)

AMX, amoxicillin; CC, clonal complex; CHL, chloramphenicol; CLI, clindamycin; CTX, ceftriaxone; ERY, erythromycin; MDR, multidrug resistant; PEN, penicillin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.

* The percentage indicates proportions of isolates carrying a macrolide resistance determinant among ERY-resistant isolates. R, resistant.

† PI, pilus islet.

‡ PEN-S, a group of six PEN-susceptible isolates.

Table 3. *Beta-lactam susceptibility and prevalence of PBP amino acid sequence alterations among serotype 19A pneumococci*

	No. (%) of isolates; MIC range (mg/l)									PBP*		
	PEN			AMX			CTX			% altered amino acids; mean (S.D.)		
CC	S (⩽0·06)	I (>0·06; ⩽2)	*R* (>2)	*S* (⩽0·5)	*I* (>0·5; ⩽2)	*R* (>2)	*S* (⩽0·5)	*I* (>0·5; ⩽2)	*R* (>2)	1a	2b	2x
Overall, *n* (%)	6 (12%)	32 (65%)	11 (23%)	21 (43%)	14 (29%)	14 (29%)	26 (53%)	19 (39%)	4 (8%)	13·9 (1·7)	7·2 (3·3)	8·7 (2·8)
CC663 (*n* = 14)	0 1·0–16	5 (36%)	9 (64%)	1 (7%) 0·5–12	11 (79%)	2 (14%)	3 (21%) 0·250–32	7 (50%)	4 (29%)	13·5 (0·8)	6·0 (1·9)	6·6 (0·7)
CC230 (*n* = 12)	0 0·094–0·5	12 (100%)	0	12 (100%) 0·047–0·5	0	0	12 (100%) 0·06–0·5	0	0	14·1 (2·0)	4·3 (0)	6·7 (1·1)
CC320 (*n* = 7)	0 1·0–2·0	7 (100%)	0	0 1·0–4·0†	1 (14%)	6 (86%)	0 1–1·5	7 (100%)	0	13·9 (1·2)	11·3 (0·3)	12·1 (0·1)
CC156 (*n* = 7)	0 1·0–4·0	5 (71%)	2 (29%)	0 1·5–8·0†	1 (14%)	6 (86%)	3 (43%) 0·5–1·0	4 (57%)	0	11·6 (1·9)	14·8 (2·0)	10·8 (2·9)
CC63, ST63 (*n* = 1)	0 0·125	1	0	1 0·047	0	0	1 0·250	0	0	9·5	3·6	11·3
Singleton, ST10433 (*n* = 1)	0 0·5	1	0	1 0·5	0	0	1 0·25	0	0	14	6·5	6·5
Singleton, ST10436 (*n* = 1)	0 2	1	0	0 2	1	0	0 1·5	1	0	14	9·4	13·1
PEN-S group‡ (*n* = 6)	6 (100%) 0·003–0·016	0	0	6 (100%) 0·01–0·032	0	0	6 (100%) 0·016–0·08	0	0	0·4 (0)	0	0

PBP, penicillin-binding protein; CC, clonal complex; MIC, minimum inhibitory concentration; PEN, penicillin; AMX, amoxicillin; CTX, ceftriaxone; S, susceptible; I, intermediate; R, resistant.

* The proportion of altered amino acids was calculated by comparing amino acid sequences of the conserved motifs of PBP1a (positions 341–582), PBP2b (positions 375–652), and PBP2x (positions 337–661) of the study isolates with those of the R6 pneumococcal reference strain. PEN-S isolates were excluded from calculations of the overall mean.

† In six out of seven isolates, an MIC for AMX was higher than an MIC for PEN.

‡ PEN-S group, a group of six PEN-susceptible isolates; described in Table 2.

Table 4. *Antimicrobial nonsusceptibility in relation to PI carriage among serotype 19A pneumococci*

	PI-negative* ($n = 20$)	PI-positive* ($n = 29$)	
Antimicrobial	No. (%) of nonsusceptible isolates		$P^{\dagger}$
PEN	14 (70%)	29 (100%)	**<0·001**
AMX	0	28 (97%)	**<0·001**
CTX	0	23 (79%)	**<0·001**
ERY	7 (35%)	22 (76%)	**0·004**
CLI	4 (20%)	16 (55%)	**0·003**
SXT	16 (80%)	17 (59%)	0·117
CHL	1 (5%)	7 (24%)	0·075
TET	16 (80%)	20 (69%)	0·390
MDR (yes)	6 (30%)	19 (66%)	**0·015**

AMX, amoxicillin; CHL, chloramphenicol; CLI, clindamycin; CTX, ceftriaxone; ERY, erythromycin; MDR, multidrug resistant; PEN, penicillin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.
* PI, pilus islet. PI-negative, isolates carrying none of the PI; PI-positive, isolates carrying PI1 or both PI1 and PI2.
† Statistically significant differences in proportions of nonsusceptible isolates among PI-negative and PI-positive pneumococci are in bold typeface.

Table S1). In addition, the prevalence of ERY- and CLI-resistant pneumococci was higher among piliated isolates (Table 4).

Sequence analyses of PBPs

Amino acid sequences of the examined PBP fragments were compared with the corresponding sequences from PEN-susceptible *S. pneumoniae* R6 strain. The number of amino acid substitutions differed between PEN-susceptible and PEN-nonsusceptible isolates, as well as between clonal groups (Table 3, Supplementary Table S2). PEN-susceptible isolates ($n = 6$) had no alterations in PBP2b and PBP2x; only one amino acid substitution was observed in PBP1a. In PEN-nonsusceptible pneumococci, the average rate of altered amino acids was 13·9%, 7·2%, and 8·7% for PBP1a, PBP2b, and PBP2x, respectively.

The rate of amino acid alteration in PBP1a was similar among different clonal groups of PEN-nonsusceptible serotype 19A isolates (Table 3, Supplementary Table S2). In 33 PEN-nonsusceptible isolates (77%), sequence alterations were found in the conserved motif $_{370}$STMK$_{373}$ (T371A/S) and in flanking amino acids of the $_{428}$SRN$_{430}$ motif (P432T) of PBP1a. In all but one PEN-nonsusceptible isolates, a four-amino acid substitution was present ($_{574}$TSQF$_{577}$ replaced by NTGY) (Supplementary Table S2).The amino acid sequence of the conserved motif $_{557}$KTG$_{559}$ of PBP1a was intact.

In PBP2b, sequence alterations were more prevalent among CC156 and CC320 isolates (Table 3). All PEN-nonsusceptible isolates had the substitution of T445A in the conservative motif $_{442}$SSNT$_{445}$ and the mutation T488A/S. All 14 AMX-resistant isolates contained a unique set of 10 amino acid substitutions (A591S, G596P, N605D, L608T, A618G, D624G, Q627E, T629N, S639T, and D640E) in the 591–640 amino acid region of PBP2b that have been associated with reduced susceptibility to AMX [33, 34]. No mutation was found in, or close to, the conserved motif $_{385}$SVVK$_{388}$ of PBP2b in any study isolate.

Similarly to PBP2b, amino acid alterations in PBP2x were more abundant in CC156 and CC320 (Table 3). The conservative motif $_{337}$STMK$_{340}$ was altered in 31 PEN-nonsusceptible isolate (72%) by the substitution T338A/P. Other prevalent mutations included I371T, R384G/S, Q552E, and N605T (Supplementary Table S2).

Next, we assessed the relatedness of amino acid sequences of PBPs of our study isolates to a number of reference sequences, constructing a neighbor-joining tree (Fig. 2). All PEN-susceptible isolates clustered together with the R6 strain (Fig. 2a–c). The amino acid sequences of PBP1a from the study isolates of CC320 were 100% identical to the reference sequences of MDR19A (ST320) and its precursor, Taiwan^{19F}-14 (ST236) (Fig. 2a). One highly resistant pneumococcus of ST10434, CC663 (Supplementary Table S1, #242; MICs: PEN and AMX, 3 mg/l; CTX, 8 mg/l) clustered to the same isolate branch. CC230 representatives were localized near this group of isolates, too. Amino acid sequences from isolates of CC156 and CC663 had an intermediate position between MDR19A (ST320) and Spain^{9V}-3 (ST156) (Fig. 2a).

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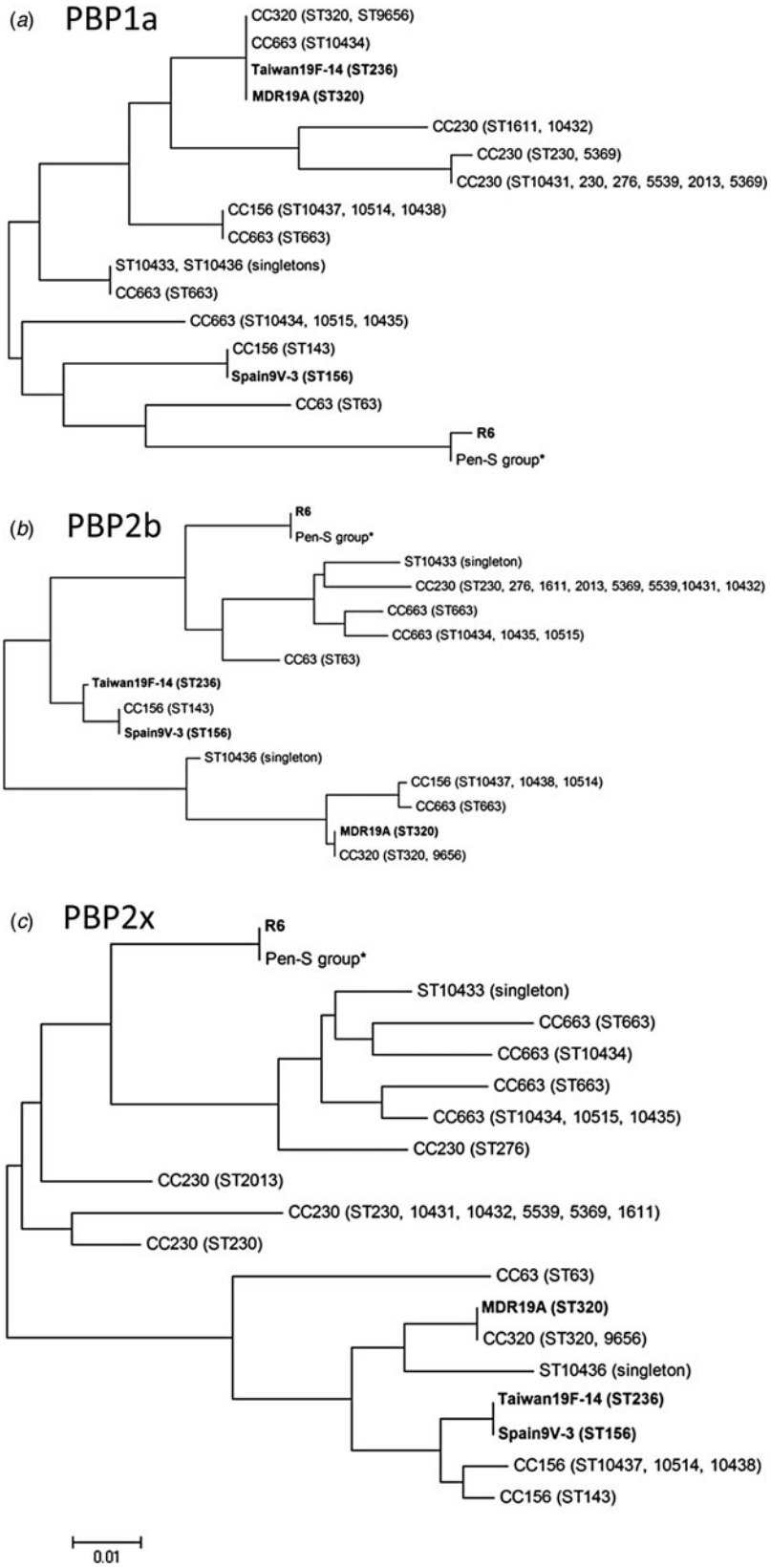

Fig. 2. Relatedness of PBP amino acid sequences among serotype 19A study pneumococcal isolates and reference strains. Sequence relatedness of PBP1a (a), PBP2b (b), and PBP2x (c) is depicted using neighbor-joining trees constructed with MEGA software. The designations of the pneumococcal reference strain R6 and clones of Taiwan^{19F}-14 (ST236), Spain^{9V}-3 (ST156), and MDR19A (ST320) are given in bold typeface. The bar represents a genetic distance of 1%. * indicates a group of 6 PEN-susceptible isolates; described in Table 2.

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For PBP2b, the amino acid sequences of CC320 and CC156 (with the exception of ST143, which was identical to Spain^{9V}-3, ST156) were equal or very similar to the MDR19A (ST320) sequences (Fig. 2b). CC230 and CC663, except for one extremely resistant isolate of ST663 (see below), comprised a big cluster of similar sequences in proximity to R6 and the PEN-susceptible group. Of note, the group of highly resistant serotype 19A pneumococci of CC156 and CC320 possessing the A681G mutation in PBP2b clustered together. One of these isolates, an isolate of ST663 with an extremely high β-lactam resistance (isolate #58, MICs: PEN, 16 mg/ml; AMX, 12 mg/ml; CTX, 32 mg/ml; Supplementary Tables S1 and S2), clustered separately from the remaining CC663 together with MDR19A (ST320) (Fig. 2b).

PBP2x sequences of CC156 were similar to those of Taiwan^{19F}-14 (ST236) and Spain^{9V}-3 (ST156) (Fig. 2c). Study isolates of CC320 and MDR19A (ST320) had fully equivalent PBP2x sequences. This group of isolates clustered separately from CC663, which was more similar to R6.

DISCUSSION

In the present study, we analyzed a set of serotype 19A pneumococci collected over 2002–2013 in several geographic locations in Russia. Four clonal groups including CC156, CC230, CC320, and CC663 composed 80% of the entire collection. These lineages related to major international clones that have different distributions over the world. CC156 has a minor representation among serotype 19A pneumococci, and this has not changed significantly under PCV pressure [6, 7, 13, 18, 21]. The same was true for the PMEN clone Colombia^{23F}-26, to which our ST663 clonal group was attributed by the eBURST analysis [6, 18]. Actually, ST663 has rarely been reported in the literature, although it was associated with high β-lactam resistance and severe IPD [35]. ST663 pneumococci had noticeable pre-PCV7 prevalence (9·6%) in Bulgaria [23]. In the present study, CC663 dominated in 2003 in European Russia. Interestingly, the earliest serotype 19A isolate of ST663 reported to the pubmlst.org/spneumoniae database, was discovered in Moscow in 1987 [16]. Thus, it is possible that ST663 and related genotypes were locally disseminated in the Eastern Europe in the 1990s and early 2000s. Of note, within the study period, we did not recover any isolate of CC199 that had high prevalence in the pre-PCV7 period in the USA and Europe [7, 18].

In contrast to the above discussed clones, CC230 and CC320 have significantly expanded in the post-PCV7 period. While the proliferation of CC230 was reported only in a few countries, e.g. Germany and Portugal [7, 8], the CC320 lineage is currently among the most prevalent serotype 19A pneumococci in many territories including the USA, Canada, Italy, and Spain [6, 18, 20, 22]. Our results demonstrated that, at present, in European Russia, CC230 and CC320 represent more than 70% of circulating 19A pneumococci; these clones have largely replaced CC156 and CC663. Pneumococci of the ST320 clonal group have been recovered in Russia starting from 2010 [26]. We found only two CC320-related STs, the identifier ST320 and the new ST9656 that is emerging in Russia. Since our collection has been assembled before the introduction of PCV in the National immunization program in 2014, the genetic structure of serotype 19A pneumococci was evolving in a PCV-independent manner, as it has been reported from some other regions [12–14].

The expansion of only a small number of serotype 19A clones implies their competitive advantage at the genotype level. Besides vaccine use, clonal success of CC320 and other serotype 19A clones is attributed to antibiotic selective pressure [17]. Indeed, in the present study we observed that all CC320 isolates had an MDR phenotype with high β-lactam MICs, which is in agreement with previous studies [6, 13, 22]. However, in European Russia, CC320 has replaced another highly resistant clone, CC663. This suggested that additional bacterial factors may be important for conferring competitive advantages. For instance, serotype 19A pneumococci of ST320 are capable of more effective colonization than their ancestral ST236 strain, at least in a murine model [14]. The increased colonization capacity of CC320 may be associated with the presence of PI1 and PI2 [10, 23, 25, 36], which have been shown to mediate adhesion of pneumococci to eukaryotic cells [30]. In the present study, CC320 was the only clonal lineage carrying both PIs, whereas CC663 isolates were PI1 positive only. Reportedly, the presence of pili is a clonal property [25, 29], and majority of antimicrobial-nonsusceptible clones does possess these structures [37]. This may suggest that piliation and antimicrobial resistance operate in concert conferring competitive benefits.

All isolates of CC156, CC320, and CC663 had PEN MICs $\geqslant$1 mg/l, whereas CC230 representatives

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had moderate-to-low PEN MICs and were fully susceptible to AMX and CTX. This indicates that β-lactam susceptibility among examined serotype 19A pneumococci has a clonal pattern. In pneumococci, the principal mechanism of β-lactam resistance is mediated through modification of PBPs [24]. Sequence analyses of penicillin-binding domains of these proteins performed in the present study demonstrated that isolates of related STs and clonal lineages had highly similar amino acid sequences explaining similar β-lactam susceptibility profiles. Clonal lineages had a variable degree of sequence alterations but most of PBP changes found in our collection were typical of those previously described. For instance, the A618G substitution close to the KTG motif of PBP2b has been previously associated with elevated AMX MICs [33, 34]. In the present study, the A618G mutation conferred an AMX MIC > PEN MIC phenotype in the majority of isolates that carried it. In addition, this mutation was found in two isolates with extremely high β-lactam MICs.

Starting from 2010, the extended-valent PCV13 that covers serotype 19A has replaced PCV7. PCV13 has demonstrated a high effectiveness in reducing the incidence of serotype 19A-related IPD [38, 39]. Being effective in the repulsion of vaccine serotypes from the circulation, vaccination with PCVs is having much broader impact on the microbial community than has been anticipated. In a recent paper by Galanis *et al*. [40], the authors demonstrated that PCV vaccination promoted expansion of new or minor pneumococcal clones and increased clonal diversity not only in vaccinees, but also in non-vaccinated individuals. These findings suggest a possibility of the spread of some serotypes in a PCV-naïve population. Most likely, the changing epidemiology of pneumococcal serotypes, including serotype 19A that we have described in the present study, reflects pressures of several factors such as vaccination and antibiotic use, as well as naturally occurring fluctuations.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0950268817000541

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DECLARATION OF INTEREST

None.

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