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Non-Typeable *Streptococcus Pneumoniae* Carriage Isolates Genetically Similar to Invasive and Carriage Isolates Expressing Capsular Type 14 in Brazilian Infants

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Abstract

Objectives—We have recently found a high prevalence of non-typeable pneumococcal isolates (NTPn) circulating in day-care centers in Central Brazil, besides serotype 14 isolates. We therefore examined the genetic relationship among NTPn and serotype 14 from carriage and invasive pneumococcal isolates obtained from children attending emergency rooms enrolled in a population-based surveillance.

Methods—The isolates were characterized by Quellung reaction serotyping, PCR for the presence of pneumolysin and the loci for a capsule gene (*cpsA*) and the type 14 gene (*cps14H*) in all NTPn, and by multilocus sequence typing and pulsed field gel electrophoresis.

Results—87.2% of the isolates were clustered into nine clusters. The major cluster included 41 pneumococcal serotype 14 (28 carriage and 13 invasive isolates) and two NTPn related to the global pneumococcal clone Spain ^{9V}-3. Overall, 95.4% of the NTPn carriage strains were genetically related to carriage or invasive strains expressing serotype 14. A dominant NTPn lineage was found, that grouped 14 pneumococcal strains. Almost half of the multidrug-resistant isolates grouped into the NTPn cluster.

Conclusion—These findings provide baseline data to assess the impact of the pneumococcal vaccination on the molecular epidemiology of *S. pneumoniae*. Changes in frequency of NTPn

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isolates and also genetic changes should be carefully monitored post vaccination, to detect potential vaccine-escape or replacement disease by capsule switched strains, especially in areas where colonization with NTPn has been frequently observed.

Keywords

molecular epidemiology; non-typeable *Streptococcus pneumoniae*; carriage; invasive pneumococcal disease

Introduction

Pneumococcal diseases are a major public health problem for young children all over the world and especially in developing countries.¹ Nasopharyngeal colonization by *Streptococcus pneumoniae* precedes invasive disease.² and evidence suggest that serotypes carried in the nasopharynx can reflect those associated with invasive pneumococcal disease in the community.^{3,4} Penicillin resistance and multidrug resistance have been gradually increasing among *S. pneumoniae* isolates and are now common worldwide, presenting in the majority of pneumococcal infections in many regions of the world.⁵ The breakpoint of the minimum inhibitory concentration (MIC) has recently changed for invasive pneumococcal disease (nonmeningitis) based on successful response to penicillin in clinical studies despite reduced susceptibility response in vitro.⁶

Studies show that day-care centers (DCCs) are ideal environments for the spread of penicillin-nonsusceptible pneumococcal (PNSp) isolates.⁷ Clones of pneumococci may differ in their ability to colonize or cause invasive disease. Great diversity in the genetic profile of carriage strains has been documented, and invasive disease strains have been associated with higher genetic similarity.^{3,8,9} Moreover, the genetic profile of PNSp strains is less diverse than that of penicillin-susceptible (PSSp) strains.^{10,11}

The vast majority of pneumococci possess a polysaccharide capsule. The capsule is the principal known virulence factor that hinders phagocytosis, and can be detected by specific antisera. Over 90 serotypes have already been recognized so far, however negative results with type-specific antisera can occur. Non-typeable pneumococci (NTPn) have usually been described as isolates optochin sensitive and bile soluble but that can not be typed with either antisera or the multiplex PCR.^{12,13,14,15} Although infrequently found as the cause of invasive disease,^{16,17,18,19} NTPn isolates have been identified as the cause of large outbreaks of conjunctivitis among healthy young people and some reports have also been shown to cause acute otitis media in infants.^{17,20,21} The prevalence of nasopharyngeal colonization with non-typeable pneumococci varies according to different regions, ranging from 0 to 11%.^{13,17,22,23,24}

In many countries, *S. pneumoniae* serotype 14 predominates among children, accounting for 50% of the PNSp isolates.^{25,26,27} In Brazil, studies have documented the emergence and rapid spread of PNSp serotype 14, which is genetically similar to the global clone Spain^{9V}-3.^{28,29} Distinction between pneumococcal clones capable of producing invasive disease and those that remain as carriers should rely on studies that analyze pneumococcal isolates from both nasopharynx carriage isolates and clinical specimens, obtained from children living in the same municipality at the same period of time.

Anticipating the introduction of a pneumococcal conjugate vaccine in Brazil, we have conducted studies on the epidemiology of both, invasive and carriage pneumococcal isolates.^{27,30} In a recent study conducted in Central Brazil, we found that serotype 14 was the main capsular type in the nasopharynx of children attending DCCs, and most of them were classified as PNSp.²⁴ In addition, we found that NTPn ranked second among all the

carriage isolates, and 62.9% of these were PNSp. In an ongoing invasive pneumococcal disease (IPD) surveillance study of children up to three years of age we have found that all serotype 14 isolates were PNSp, comprising 50% of all isolates.³¹ The majority of children with IPD were outpatient pneumonia and were treated with oral ampicillin.³²

These findings prompted us to assess the genetic similarity between serotype 14 and NTPn nasopharyngeal isolates, as well as to compare these with all serotype 14 invasive isolates from the ongoing IPD population-based surveillance in the same pediatric population. Therefore, in this study, a total of 133 pneumococcal strains were submitted to molecular analysis. Knowledge of the genetic characteristics of the main pneumococcal strains circulating in the community is needed in order to establish a robust baseline prior to vaccination.

Methodology

Obtaining carriage and invasive pneumococcal strains

Surveillance study in day-care centers—As part of the ongoing effort to assess the potential impact of the pneumococcal conjugate vaccine in Brazil, we conducted surveillance of pneumococcal nasopharyngeal (NP) carriage among children who attended DCCs in the city of Goiânia in central Brazil. Details of this survey are described elsewhere.²⁴ Briefly, between August and December 2005, a cross-sectional study on NP carriage of PNSp was carried out among 1,192 children between the ages of 2 and 59 months attending 62 DCCs in the municipality. The study protocol was approved by the Regional Ethical Committee from the Federal University of Goiás (UFG), and written informed consent was obtained from each child's parents or legal representative. A single pernasal, extra-thin, flexible calcium alginate nasopharyngeal swab sample was obtained per child (Transwabs, Medical Wire, Corsham, UK), following the World Health Organization guidelines for collection and transport of NP samples.³³ Among 166 PNSp isolates, the majority of typed isolates were determined to be serotype 14 (53%), with the next largest group found to be NTPn (13.3%).

Surveillance of invasive pneumococcal disease (IPD)—Invasive isolates were obtained through an active, prospective, population-based IPD surveillance launched in 2007 among children up to three years of age. The methodology and preliminary results of the surveillance were previously reported.³¹ In summary, children attending emergency rooms with temperature $\geq 39^{\circ}\text{C}$ and/or clinical suspicion of pneumonia, meningitis, sepsis or other IPD (e.g., arthritis, osteomyelitis or peritonitis) were invited to participate. The surveillance project was approved by the Regional and National Brazilian Ethical Committees, and informed consent was obtained from the children's parents. IPD was defined as isolation of *S. pneumoniae* from a normally sterile site (e.g., blood, pleural fluid or cerebrospinal fluid). Only one isolate from each child was included in the analysis. Pneumococcal serotype 14 accounted for 59.4% of the PNSp invasive isolates obtained between May 2007 and December 2008. These samples were used as a comparison group for the type 14 PNSp isolates obtained in the DCC carriage survey.

***S. pneumoniae* strain characterization**—Pneumococcal isolation and identification was performed at the Bacteriology Laboratory at the UFG and at the Public Health Laboratory of Goiás State (LACEN), for carriage and invasive isolates, respectively, by standard methods. Isolates of *S. pneumoniae* were subcultured for antimicrobial susceptibility testing. All isolates were screened for susceptibility to oxacillin by disk diffusion (OXOID, Basingstoke, England), and strains with diminished susceptibility to penicillin (halo ≤ 19 mm) were analyzed for minimum inhibitory concentrations (MICs)

using Etest® (AB BIODISK, Solna, Sweden). Analysis of susceptibility to ceftriaxone, erythromycin, trimethoprim-sulfamethoxazole (TMP-SMZ), levofloxacin, tetracycline, clindamycin, chloramphenicol, rifampicin and vancomycin was routinely performed by the disk-diffusion method and confirmed by the Etest®. The interpretation of antimicrobial susceptibility followed the Clinical and Laboratory Standards Institute (CLSI).³² The rationale for penicillin MIC breakpoints was conceived to guide penicillin therapy for patients with invasive pneumococcal disease isolates, as pneumococcal isolates derived from asymptomatic carriage children are not supposed to be treated.

S. pneumoniae ATCC 49619 was used for quality control of the disk-diffusion assays and as an internal control in the susceptibility tests. Multidrug-resistant (MDR) *S. pneumoniae* was defined as a strain nonsusceptible to at least three different antimicrobial classes.

Serotyping of isolates

Pneumococci were serotyped at the IAL by Quellung reactions using standard antisera obtained from the Statens Seruminstitut (Copenhagen, Denmark).³⁴ For carriage isolates, all NTPn isolates and 24% of isolates expressing serotype 14 were tested for capsular type by a multiplexed inhibition immunoassay for capsular polysaccharides (multibead system), as previously described.^{35,36} Bead set 1 contained beads conjugated with polysaccharides (PSs) of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9N, 9V, 11A, 14, 18C, 19A, 19F and 23F. Bead set 2 contained beads conjugated with PSs of serotypes 2, 6A, 8, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F.

Polymerase chain reaction (PCR) for genetic studies

NTPn carriage isolates and a sample of serotype 14 isolates were examined for the presence of pneumolysin (*ply*), *lytA*, *cpsA* and *cps14H* genes by PCR. The presence of pneumolysin or *lytA* indicates that the isolates are *S. pneumoniae*, while the presence of *cpsA* shows that the isolates have the capsule gene locus of any serotype. The *cps14H* gene was used to verify whether the isolates had the type 14 gene locus, which may be either functional or non-functional. PCR mixtures were incubated at 94°C for 10 min for initial denaturation. Then, the samples were subjected to 30 thermal cycles. The conditions included denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. PCR products were separated by electrophoresis in 1% agarose gels and visualized with ethidium bromide under an ultraviolet light. The sequences of the primers used were 5'-ATTTCTGTAACAGCTACCAACGA-3' for pneumolysin forward, 5'-GAATTCCTGTCTTTTCAAAGTC-3' for pneumolysin reverse, 5'-CTTGCGCAGGTGTCAGAATTCCTCTAC-3' for *cps14H* forward, 5'-GCCAAAATACTGACAAAGCTAGAAATATAGCC-3' for *cps14H* reverse, 5'-GCAGTACAGCAGTTTGTGGACTGACC-3' for *cpsA* forward and 5'-GAATATTTTCATTATCAGTCCAGTC-3' for *cspA* reverse. The *lytA* primers were 5'-TTATTCGTGCAATACTCGTGCG-3' for the reverse primer and 5'-CAACCGTACAGAAATGAAGCGG-3' for the forward primer. (Dr Moon – is it possible to replace the sequence of the primers by a reference?)

Pulsed-field gel electrophoresis (PFGE)

The methodology for PFGE was adapted from a previously described protocol.³⁷ Genomic DNA was prepared in situ in agarose blocks and digested with 20 U of *Sma*I (Invitrogen Corporation, Carlsbad, CA). Restriction fragments were separated by PFGE on a CHEF DR-II apparatus (Bio-Rad Laboratories, Birmingham, UK) with the following running parameters: initial pulse 5 s; final pulse, 35 s; field strength 6 V/cm for 22 h at 11°C. Lambda DNA ladder (New England Biolabs, EUA) was used as a molecular weight standard. Pneumococcal strain R6 (GenBank accession number NC003098, kindly provided

by Dr. Herminia de Lencastre from the Institute of Chemical and Biological Technology, Oeiras, Portugal. Gels were stained with ethidium bromide and photographed with a photo-gel system (Quality One Program, version 4.5, Universal HOOD II, Gel Doc EQ, Bio-Rad). PFGE band patterns were first assigned by visual inspection of the macrorestriction profiles following the Tenover criteria,³⁸ and further analyzed using BioNumerics software (Applied Maths, version 5.01, Belgium).

Multilocus sequence type (MLST)

The multilocus sequence types of the *S. pneumoniae* isolates were identified as described elsewhere. Briefly, PCR was performed as above for the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* genes using primers that were previously described.³⁹ The resulting PCR products were sequenced at the UAB.

Analysis

Gel photos were scanned and imported into a BioNumerics database as inverted 8-bit gray-scale TIF images. The dendrogram was constructed by means of the Dice band-based similarity coefficient, with a band position tolerance of 1.5% and an optimization of 0.7%. Isolates were defined as epidemiologically related if they shared 80% similarity on the dendrogram. A cluster was defined as three or more related isolates. PFGE fingerprint patterns were compared with those of 26 available global pneumococcal clones⁴⁰ stored at the IAL. Comparisons between penicillin MICs were determined using the Kruskal-Wallis analysis of variance test.

Results

Table 1 shows details of the two studies from which the carriage and invasive pneumococcal isolates were obtained. Among the 133 PNSp isolates submitted to PFGE, 106 were obtained from NP samples of DCC attendees, and 27 were from invasive infections. Of the 106 carriage isolates, 84 were serotype 14, and 22 were NTPn isolates. All 27 invasive strains were serotype 14. Multibead assay and Quellung reaction agreed in all pneumococcal capsular type, except for one NTPn strain for which multibead was able to discriminate it as 19F capsular type. MIC values for penicillin were significantly higher for invasive than for carriage isolates ($p < 0.05$).

Based on PFGE analysis, 116 out of 133 strains grouped into nine clusters (Table 2 and Figure 1), while 16 pneumococcal strains did not group; these included 12 carriage serotype 14 isolates, three invasive serotype 14 isolates and one NTPn strain. Three main pneumococcal clusters were identified. Cluster 1 included 41 serotype 14 strains (28 carriage and 13 invasive strains) and two NTPn carriage strains, which were highly related to the global pneumococcal clone Spain^{9V}-3. Cluster 2 was composed by 29 serotype 14 and two NTPn isolates. Cluster 3 grouped the majority of NTPn carriage strains, which were genetically related (81.8%) to three carriage serotype 14 isolates. A higher prevalence of MDR strains was found in the NTPn cluster (cluster 3, 47.4%) as compared to cluster 1 (20.9%) and cluster 2 (6.4%). Overall, 21 of the 22 NTPn carriage strains (95.4%) were genetically related (> 80%) to carriage or invasive strains of pneumococcus expressing capsular type 14. Molecular analysis of the NTPn isolates by PFGE revealed a high degree of diversity (54.5% similarity), although a dominant lineage that grouped 14 pneumococcal strains (83.6% similarity) and a second lineage that grouped only three isolates could be identified (data not shown).

The molecular analysis shown in Table 3 demonstrates that 86.4% of NTPn strains did not have the capsule gene but were genetically related to capsular type 14 isolates (Figure 1).

Three NTPn strains (13.6%) had the capsule gene locus specific to serotype 14, and two of them were ST66, a sequence type found among pneumococcal strains from meningitis patients in Brazil. According to the MLST database, NTPn nasopharyngeal strains #225 and #536 were related to clone Spain^{9V}-3 (Table 3 and Figure 1), as they belonged to PFGE type A.

Discussion

This study provides solid evidence that several pneumococcal strains colonizing the nasopharynx are genetically related to the invasive strains currently causing pneumococcal disease in the pediatric population in our setting. In a previous report we found that strains colonizing the nasopharynx were responsible for IPD in the community.⁴¹ Hathaway and colleagues¹³ evaluated 27 non-encapsulated pneumococci and found evidence that NTPn isolates were genetically related to encapsulated strains, especially capsular types 33F and 37. Our findings indicate that the NTPn strains are genetically similar to invasive and carriage strains expressing capsular type 14. In contrast, Sá-Leão and co-authors¹⁵ examined a collection of 213 NTPn isolates and observed that their genetic background differed from those found in serotypeable strains.

We detected a dominant lineage of pneumococci expressing capsular type 14 that was genetically similar to the international Spain^{9V}-3 clone. This lineage congregated with the majority of the carriage and IPD pneumococcal strains. The penicillin-nonsusceptible Spain^{9V}-3 clone, first identified in Spain and France, has become widely disseminated throughout the world and has been identified in several countries in South America.⁴⁰ Serotype variants of the Spain^{9V}-3 clone have been reported in a number of countries, but serotype 14 isolates are the most commonly encountered strains.^{42,43} This clone is highly prevalent in Brazil; it was first reported in the mid-1990s in Brazilian children with IPD²⁸ and more recently reported in the nasopharynx of Brazilian adults with human immunodeficiency virus.⁴⁴ We also found that pneumococcal isolates related to Spain^{9V}-3 clone presented higher penicillin MICs (many $\geq 2 \mu\text{g/ml}$). Spain^{9V}-3 is probably the clone responsible for the spread of resistance to penicillin in our municipality, as observed in other places of Latin America.⁴⁵

Another interesting observation is that a large number of nasopharyngeal isolates were not typeable and may not produce capsules. For example, in our study, 19 out of 22 isolates with ST2315 lacked both the *cpsA* and *cps14H* genes; and these isolates may produce no capsular polysaccharide at all. Other investigators have also reported non-typeable pneumococcal carriage isolates without *cpsA*¹⁴ including those expressing ST2315 in Brazil (www.mlst.net). Thus, it is likely that humans can carry pneumococci that cannot produce capsules. This epidemiologic observation is surprising because experimental studies have shown that production of some capsular polysaccharide is important for experimental colonization of the mouse nasopharynx with pneumococci as the capsule helps pneumococci avoid mucus-mediated clearance even though excessive amount of capsule may interfere with colonization.^{46,47}

It is also quite possible that some of the apparently non-typeable pneumococcal isolates may produce capsules in small amounts that cannot be detected with the standard Quellung reaction or the multibead assay. These assays were designed for capsule-rich isolates from IPD patients and have low sensitivity. Thus, strains with the *cps14H* gene should be carefully examined for production of capsules. However, even these strains may not produce capsules at all, since biofilm formation was found to be important for nasopharyngeal carriage and pneumococci maintained under biofilm-promoting conditions tend to become acapsular, often via inactivation of the *cpsE* gene.^{48,49} Perhaps humans, unlike mice in

pneumococcal disease models, can carry pneumococci for a lengthy period of time, in which some pneumococci may become acapsular.

Following the introduction of PCV vaccination in many setting, the number of invasive NTPn isolates has remained essentially unchanged.^{16,19} Nevertheless, a different scenario has been encountered in day-care centers. A good example is the study by Sá-Leão and colleagues, who observed a 3.4-fold increase in NTPn strains after pneumococcal vaccination compared to the pre-vaccination period.²³ Thus, the potential role of non-typeable isolates in pneumococcal disease and serotype replacement is currently an open question. As we showed that most non-typeable isolates were related to serotype 14 strains isolated from invasive disease, including the Spain^{9v} clone, we could speculate that carriage of non-typeable strains would serve as a reservoir for future replacement disease by capsule switched strains. Another concern is that we have found NTPn carriage strains highly associated with penicillin nonsusceptible isolates,²⁴ similarly to what has been described by others,²³ placing non-typeable isolates as a hidden source for spreading out nonsusceptible isolates, not covered by pneumococcal conjugate vaccines.

In June 2010, Brazil introduced the 10-valent pneumococcal conjugate vaccine routinely into its national immunization program. Loss of capsular genes may provide NTPn with a selective advantage and monitoring the prevalence of such isolates, would be of interest to follow changes in frequency of NTPn, and also genetic changes post vaccination, as a proxy of a possible vaccine-escape, in areas where colonization with NTPn has been frequently observed. Whether pneumococcal vaccine would give some protection against NTPn genotype 14 is an interesting issue that requires further investigation.

In conclusion, epidemiologic and molecular evidence show that PNSp isolates of serotype 14 circulating in Goiânia are genetically similar to clone Spain^{9v}-3 and play a critical role in the spread of PNSp strains to the entire pediatric community of Goiânia. Also, NTPn isolates are genetically related to carriage and invasive strains of pneumococcus expressing capsular type 14. Altogether, these findings provide invaluable baseline data to assess the impact of the pneumococcal vaccine on the molecular epidemiology of *S. pneumoniae* in the community.

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Table 1
Characterization and origin of *Streptococcus pneumoniae* isolates submitted to genetic analysis

Variables	Carriage isolates	Invasive isolates
Study design for obtaining strains	Period prevalence ^a	Active population-based surveillance ^b
Study period	August to December, 2005	May 2007 to December 2008
Study population	Health children attending 62 DCC ^c	Children with suspicion of IPD ^d attending 33 ER ^e
Age-group	3-59 months	28 days up to 36 months
Population-at-risk	3,720 day-care attendees	Cohort of 52,998 children 36 months
PNSp ^f /pneumococcal isolates	178/648	40/60
Serotype 14/no. of serotyped	88/166	29/60
Serotype 14 submitted to PFGE ^g /no. of serotyped	84/88	27/29
Non-typeable submitted to PFGE ^g /non-typeable	22/22	-
MIC to penicillin - median (variance)	1.00 ^h (1.028)	2.00 ^h (1.901)
Total pneumococcal strains submitted to PFGE ^g	106	27

^aFranco et al, 2010²⁴

^bAndrade et al, 2008

^cDay-care centers

^dInvasive pneumococcal disease

^eEmergency rooms

^fPenicillin nonsusceptible pneumococcal

^gPulsed-field gel eletrophoresis

^hKruskal Wallis, p value = 0.033

Table 2

Characteristics and genetic similarity by PFGE^a of pneumococcal clusters according to carriage and invasive strains and capsular type.

Clusters	No. of grouped pneumococcal strains (N=116)	Serotype 14		NTPn ^b carriage (N=21)	% of genetic similarity	Tenover classification ^c
		Carriage (N=72)	Invasive (N=23)			
1	43	28	13	2	81.8	A-A22; Span ^{9V} -3
2	31	22	7	2	81.3	B-B11
3	19	3	-	16	81.8	C-C11
4	5	4	1	-	81.5	D-D2
5	4	3	-	1	84.1	E-E3
6	4	4	-	-	80.1	F-F3
7	4	4	-	-	94.7	G-G1
8	3	1	2	-	82.6	H-H1
9	3	3	-	-	87.0	I-I1

^aPulsed field gel electrophoresis

^bNTPn = non-typeable pneumococcal

^cTenover et al. 1995

Table 3
Phenotype and genotype characterization of non-typeable pneumococcal isolates from day care attendees.

Strain	Serotype	Capsule genes				MLST ^a allele number							ST	Source ^b	Related global clone
		<i>ply</i>	<i>lytA</i>	<i>cpsA</i>	<i>cps14H</i>	<i>aroE</i>	<i>ghd</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>			
231	NTPn ^c	+	+	-	-	8	37	9	12	2	28	224	2315	BRA ^d , nasopharynx	
264	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
254	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
30	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
730	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
523	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
731	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
926	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
869	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
261	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
452	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
542	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
297	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
834	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
693	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
225	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	clone Spain ^{9V} -3
569	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
156	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	
536	NTPn	+	+	-	-	8	37	9	12	2	28	224	2315	BRA, nasopharynx	clone Spain ^{9V} -3
1176	NTPn	-	+	+	+	1	48	4	1	10	1	8	DLV7 ^f	UK, AUS, CSF, blood	
131	NTPn	+	+	+	+	2	8	2	4	6	1	1	66	BRA, CSF	
139	NTPn	+	+	+	+	2	8	2	4	6	1	1	66	BRA, CSF	

^aMultilocus sequence type

^bClinical specimen of the related ST strain, as reported at the database at www.mlst.net

^cNon-typeable isolates

d_{Brazil}

$e_{\text{Cerebrospinal fluid}}$

$f_{\text{Double locus variant of sequence type 7}}$