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ORIGINAL ARTICLE

Multiplex PCR to *Streptococcus pneumoniae* serotype identification directly in cerebrospinal fluid samples

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Abstract

Streptococcus pneumoniae causes invasive diseases of significant public health concern, such as meningitis. The culture of cerebrospinal fluid (CSF) samples, the standard technique for meningitis diagnoses, is not always positive. Consequently, meaningful information about the etiological agent is lost, which can compromise effective epidemiological surveillance and the improvement of immunization policies. This study aims to standardize a method to genotype pneumococcus in the CSF samples which could mitigate the absence of isolated strains, and also evaluate the prediction of this assay. We applied eight multiplex PCR (mPCR) assays to CSF samples paired with the Quellung reaction applied to the isolated strains. We also compared different master mix kits in the mPCR. Moreover, a retrospective study was conducted with CSF samples considered pneumococcus positive due to the presence of the *lytA* gene. Results showed that genotyping by the mPCR correlated 100% with the Quellung reaction, and genotyping was dependent on the master mix applied. In the retrospective study (2014–2020), 73.4% were successfully genotyped. The analyses of the receiver operating characteristic curve showed that the cycle threshold (Ct value) around 30 for the *lytA* gene had a 75% positive chance of successful genotyping, whereas with a Ct value > 35, the chance was 12.5%. Finally, we observed that genotype 19A was prevalent in the period (12%), information unknown until now due to the lack of isolated strains. Therefore, the mPCR of CSF samples can efficiently predict *S. pneumoniae* serotypes, especially in the absence of isolated strains, which can be a great tool for pneumococcal serotype surveillance.

Keywords Molecular diagnostic · Multiplex PCR · Pneumococcal meningitis · Pneumococcal serotypes · Cerebrospinal fluid · Serotype surveillance

Introduction

Pneumococcal meningitis, caused by *Streptococcus pneumoniae* bacterium (also called pneumococcus, or *S. pneumoniae*), is generally an endemic disease, and it can potentially cause large community outbreaks [1]. The culture of cerebrospinal fluid (CSF) samples is the gold standard for diagnosis of meningitis [2]; however, the low bacterial growth rates particularly in the patients who have received prior antibiotic treatment bring the necessity to develop new tests [3]. PCR-based assays to detect pneumococci from clinical samples do not require viable bacteria and present very high sensitivity, stating an important tool in the diagnosis of invasive pneumococcal infections [4, 5]. Serotype distribution can be determined by the culture of the microorganism followed by the determination of the capsular type by individual serotype-specific antisera binding known as Quellung reaction [2]; however, it is a time-consuming and high-cost test. Thus, pneumococcal serotyping using molecular methods directly on clinical samples can be cheaper and faster; also, it can be a great tool in pneumococcal serotype epidemiological surveillance, especially in the absence of isolated strains. A broth enrichment approach to isolate pneumococcal strains from

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nasopharyngeal specimens to determine serotypes from pneumococcal carriage by using eight multiplex PCR conventional was standardized by the Centers for Disease Control and Prevention, USA (CDC) [6]. They applied DNA obtained from isolated strains to predict a great variety of serotypes, and the results were compared to the results of conventional culture-based. This PCR-based strategy that employed a broth enrichment step appeared to enhance the detection of mixed serotypes and low-density pneumococcal carriage [6], whereas Saha et al. [7] optimized a multiplex PCR (mPCR) scheme to identify only seven capsular serotypes likely to occur in Bangladesh, and that could be performed not only with isolated strains but also directly from CSF samples of culture-negative cases. It is not feasible to perform the Quellung method in culture-negative cases due to the absence of isolated strains, thus mPCR can overcome this issue. Also, the mPCR can be helpful in the epidemiological evaluation of circulating serotypes and, consequently, in making accurate decisions about vaccination. This approach can also be useful in population-based studies where there is limited laboratory capability for taking on the highly demanding work of conventional pneumococcal isolation and serotyping [8].

More than 100 different pneumococcal serotypes have been described [9] and it has been reported that serotype distribution of the pneumococcus varies due to many factors such as clonal expansion, capsular transformation, mass pneumococcal vaccination, socioeconomic settings, immune status, and antibiotic use in the population. Moreover, it is expected that about a third of them are implicated in pneumococcal disease [10]. Numerous assays have been described in which serotype/serogroup-specific PCRs have been developed and multiplexed, in different combinations, to identify a variable number of commonly occurring serotypes [10]. Two reported studies identified most of the targeted serotypes individually or to serogroups and excluded isolates belonging to other serotypes [11, 12]. Molecular methods are also helpful, even after mass vaccination starts, in monitoring possible epidemiological changes in serotype distribution, especially for meningitis in settings with high prior use of antibiotics [7, 13]. Although serotyping of pneumococci is not usually necessary for a clinical response, capsular serotype determination is a critical component of successful pneumococcal disease control, particularly in the aspect of vaccine development and vaccination strategies. Thus, the main goal of the present study was to evaluate the mPCR for *S. pneumoniae* serotypes identification directly from CSF samples, without culture procedures and DNA purification from pneumococcus isolates, based on a modification of the CDC's standardized eight multiplex PCR conventional, which can deduce about 70 pneumococcal serotypes prevalent in Latin America [6]. Moreover, we also presented here the description of the meningitis etiology obtained by culture and qPCR techniques during the studied period and their comparison.

Materials and methods

Samples

From 2017 to 2020, CSF samples from patients suspected of bacterial meningitis were received for diagnostic confirmation purposes in the Adolfo Lutz Institute – Regional Laboratory of Santo André, which is the public health laboratory in the region of seven cities in the Greater Sao Paulo, Brazil. Firstly, the samples had the DNA extracted by heating (100 °C for 5 min) in a dry block. Then, multiplex qPCR was performed as standardized at Adolfo Lutz Institute [5, 14] to detect the following genes: *ctrA* from *Neisseria meningitidis*, *lytA* from *Streptococcus pneumoniae*, and *hpd* from *Haemophilus influenzae*, as part of the diagnosis routine of our laboratory. When there was enough CSF sample volume, 125 µL were also plated on chocolate blood agar plates and incubated in an anaerobic jar at 36 °C for 48 h, as a new attempt to isolate any microorganism since diagnosis laboratories from hospitals have already performed the culture in the first moment after collection.

Multiplex PCR standardization

The eight multiplex PCR conventional, which were previously standardized by CDC with purified genomic DNA from isolated strains [6], were adapted in this study to be performed with CSF samples. For this purpose, each reaction was firstly tested with different annealing temperatures and different final volumes using different commercial master mixes kits: 25 µL for QIAGEN® Multiplex PCR Kit (Qiagen, Germany), as standardized by Carvalho et al. [6]; 50 µL for Platinum® Multiplex PCR Master Mix (Thermo Fisher Scientific, USA); 95 µL for Invitrogen™ PCR Supermix Brasil (Thermo Fisher Scientific); 25 µL for GoTaq® Hot Start Green Master Mix (Promega, USA); and 50 µL 2×PCR SuperMix (Bio-Helix, Taiwan). The concentration of the 12 primers presented in each of the eight multiplex PCR, summarized in Supplement 1, was adjusted for the different volumes of each master mix, according to Carvalho et al. [6]. The PCR protocol was executed agreeing to kit manufacturer specifications, using 54 °C as the annealing temperature. Only when the QIAGEN® Multiplex PCR Kit was applied the annealing temperature was different for each one of the eight multiplex PCR: reactions I and VIII was 59 °C; reactions II, III, IV, V, VI, and VII were 57 °C. The Veriti™ 96-Well Thermal Cycler was used with the standard 0.2 mL block format (Thermo Fisher Scientific) for all

reactions. PCR products were electrophoresed in TAE buffer with a 3% agarose gel stained with 1:10,000 Invitrogen™ SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific), at 70 V for 80 min. Invitrogen™ BlueJuice™ Gel Loading Buffer (10×) was added to the sample and Invitrogen™ 100 bp DNA Ladder (both Thermo Fisher Scientific) was used as molecular weight. Then, the gel was visualized and the image was captured by E-Gel Imager—Gel photo documentation system and the GelCapture Software—Image Acquisition (both Thermo Fisher Scientific).

In addition, the master mixes were tested with selected sample templates, categorized as A1—high pure DNA purified from isolated strain (serotype 22F); A2—high-quality DNA extracted from CSF sample, (serotype 8); A3—low-quality DNA extracted from CSF sample (serotype 22F presented in the human sample which strain used in the A1 was isolated). Then, the amounts of amplicons were quantified after agarose gel by GelQuant Express—Image Analysis software from Thermo Fisher Scientific, to check the molecular weight in nanograms (ng) and bases pair (bp).

Comparison of methodologies

After the standardization of the eight multiplex PCR using CSF samples, all *S. pneumoniae* culture-positive CSF samples were submitted to the eight multiplex PCR to detect capsules pneumococcal genes. The results were compared to the Quellung reaction performed with isolated strains. Moreover, the same protocol was applied to all *lytA*-positive CSF samples received between 2014 and 2020, independently if there was an isolated strain, to produce a retrospective study. In this regard, CSF samples with great volumes were submitted to another genomic DNA extraction using silica column kits (PureLink™ Genomic DNA Mini Kit—Thermo Fisher Scientific, USA or QIAamp® DNA Mini Kit – Qiagen, Germany), to concentrate the DNA. Then, the profile of pneumococcal strains causing meningitis in the region was evaluated.

Statistical analyses

The statistical analyses were performed using R Statistical Software (version 4.1.0). Logistic regression was calculated based on the variable Ct value obtained for the *lytA* gene in qPCR and other variables such as the success of identification of the genotype by the eight multiplex PCR; master mix tested; patient outcome (death or not); the age of the patient; city of residence; type of DNA extraction (silica column kit or heating); quantity of detected bands (0, 1, or 2); the result of cultivation (growth or not); and the genotype identified by the eight multiplex PCR. A model of prediction of the outcome of the eight multiplex PCR was established when CSF samples were applied. Also, chi-square and Fisher tests were applied to compare the categorical variables and the significance was considered with a p-value < 0.05. Mann–Whitney test and Student's t-test were applied to compare the Ct value obtained for the *lytA* gene in qPCR and the culture growth, or the success of genotyping identification, respectively. The mean and standard error (SE) was also presented. These both were calculated using the GraphPad Prism version 5.0. In addition, a receiver operating characteristic (ROC) curve was designed to show the true positive percentage and the false positive percentage for the possibility of genotype identification.

Table 1 Comparison of culture and qPCR techniques. Number of samples and percentage

Culture performed $n = 263$	Culture-positive 72/263 (27.4%)	Result qPCR = culture 38/72 (52.8%)	*Streptococcus pneumoniae*	26/72 (36.1%)
			Neisseria meningitidis	10/72 (13.9%)
			Haemophilus influenzae	02/72 (2.8%)
		Result qPCR ≠ culture 34/72 (47.2%)	qPCR and culture-positive but discordant	02/72 (2.8%) [b]
			qPCR negative and culture-positive to S., N., or H. [a]	01/72 (1.4%) [c]
			qPCR invalid and culture-positive	01/72 (1.4%) [d]
			qPCR negative and culture-positive different species	30/72 (41.7%)
	No bacterial growth 191/263 (72.6%)		qPCR = culture (both negative)	127/191 (66.5%)
			qPCR invalid	01/191 (0.5%)
			qPCR positive and culture-negative	63/191 (33.0%) [e]

[a], *S.*: *Streptococcus pneumoniae*; *N.*: *Neisseria meningitidis*; *H.*: *Haemophilus influenzae*; [b], CSF sample with qPCR positive to *Neisseria meningitidis*, but culture-positive to yeast and *Streptococcus* sp; [c], qPCR negative, but culture-positive to *Haemophilus influenzae*; [d], qPCR invalid, but culture-positive to *Staphylococcus* sp; [e], bacteria not isolated but detected in the qPCR, thus dead strains

Results

qPCR and culture comparison

From August/2017 to September/2020, 377 clinical CSF samples were received in our laboratory for bacterial meningitis investigation. The DNA of all samples was extracted for qPCR performance but 263 samples that had higher volumes were used in the culture. Bacterial growth was observed in 72 samples (27.4%) and 26 of those samples were culture-positive for pneumococci (36.1%). Culture and qPCR had 52.8% (38 samples) of results compatible, as presented in Table 1. Moreover, from 191 negative cultures (72.6%), 63 were qPCR positive, indicating that at least 33% of CSF samples had unviable microorganisms when the clinical specimens were received.

According to the results of qPCR, it was possible to identify the recovery percentage of strains. Fifty positive results were observed in qPCR for pneumococcus; however, only 26 samples were culture-positive, indicating 52% recovery. Other bacteria such as *N. meningitidis* and *H. influenzae* had 22.2 and 22.7% recovery, respectively. These data are summarized in Supplement 2. Moreover, when evaluating the cycle threshold (Ct value) of the multiplex qPCR for the *lytA* gene and the positivity of the culture (Fig. 1), an association was observed. The Ct values of culture-positive samples were significantly lower (mean $21.12 \pm$ SE 0.83), $p < 0.0001$, than the Ct values of culture-negative samples, which presented higher Ct values (mean $29.63 \pm$ SE 1.07). This suggests that in samples with lower Ct values, there is higher DNA concentration and consequently more bacteria in the sample.

Different master mixes applied for the multiplex PCR

The eight multiplex PCR were initially tested with different master mix kits. With the aim to select the master mix with the best cost–benefit, the amount of amplified DNA from the same template applied for each master mix was quantified. Extracted DNA from cultured strain as a template which was considered high pure DNA quality was tested, and also from CSF samples considered here with different qualities: low quality, due to the difficulty to genotype with all master mixes tested, and high quality, due to the low Ct value (= 19) and the ability to genotype with all master mixes tested. The success of the reaction was measured by the detection of two bands in the agarose gel (one referring to the genotype group and the other to the *cpsA* gene, the positive control) and by the quantification of these bands. Figure 2 shows that the master mix which produced a better yield of amplicon for all templates tested was QIAGEN® Multiplex PCR Kit and GoTaq® Hot Start Green Master Mix (lanes 10–12 and 13–15 respectively). The master mix 2 × PCR SuperMix (Bio-Helix) presented the best yield for DNA from the isolated strain (lane 7), however, presented the worst result for the low-quality CSF sample. Moreover, it seems that presented high levels of unspecific bands (dot arrow). It is expected not to be primers in excess since the same concentration was applied for all reactions. Additionally, we ranked the master mixes according to this quantification and thus their sensitivity observed in this assay: 1st QIAGEN® Multiplex PCR Kit; 2nd GoTaq® Hot Start Green Master Mix (Promega); 3rd 2 × PCR SuperMix (Bio-Helix); 4th Invitrogen™ PCR

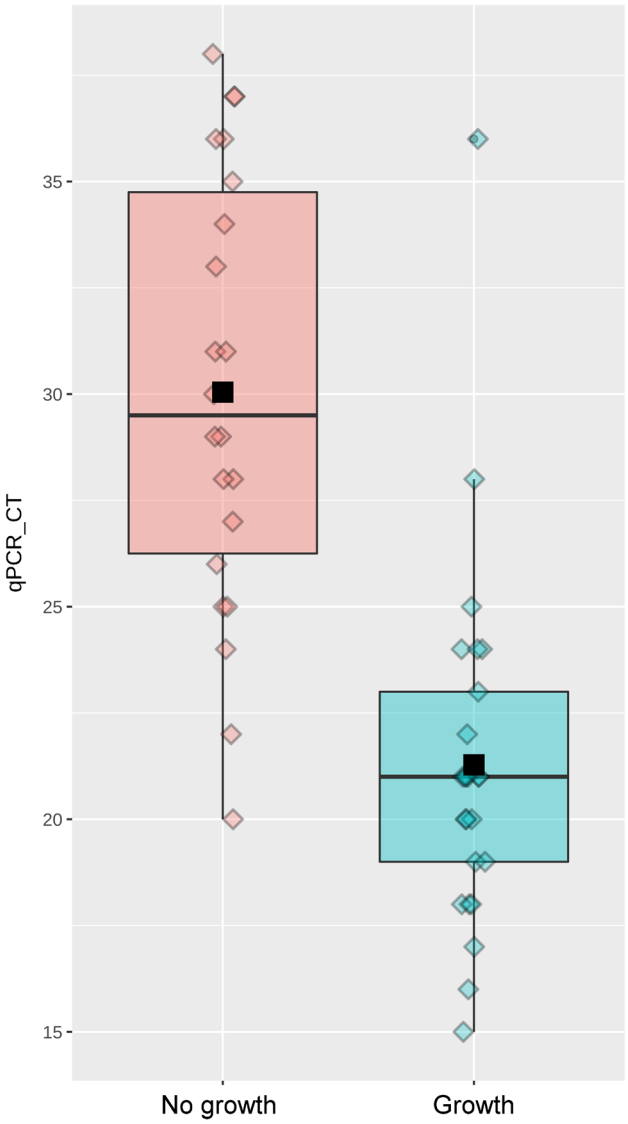


Fig. 1 Cycle threshold for the *lytA* gene of the bacterial meningitis multiplex qPCR (qPCR_CT) versus the results of CSF samples culture for the growth of *Streptococcus pneumoniae*. Each diamond represents a sample. The black bar represents the median and the black square represents the mean

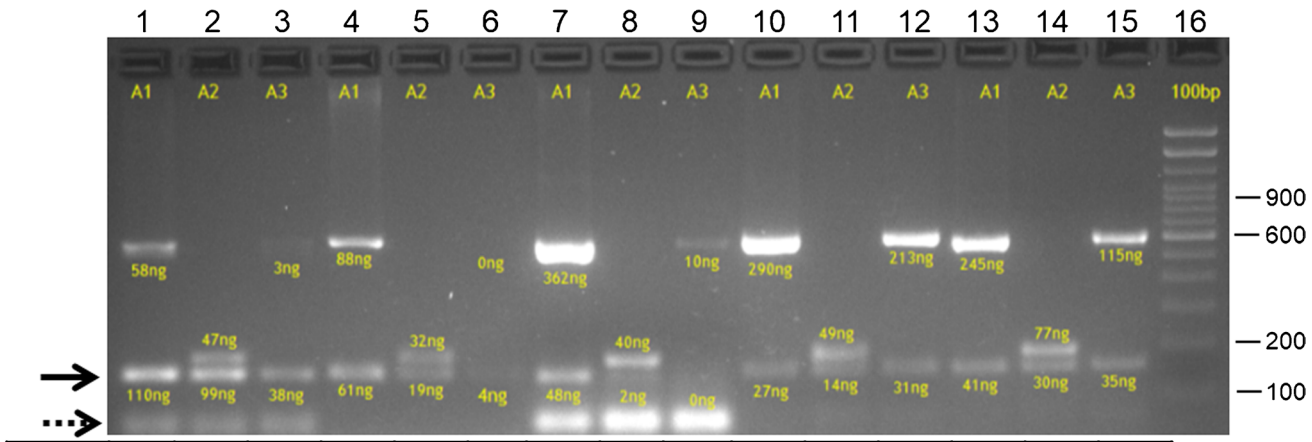
Supermix Brasil (Thermo Fisher Scientific); 5th Platinum® Multiplex PCR Master Mix (Thermo Fisher Scientific).

Quellung versus multiplex PCR

From 26 CSF samples that were isolated *S. pneumoniae*, 25 strains had DNA purified for the performance of the eight multiplex PCR, and also they were tested in the Quellung reaction because one strain was not viable in further culture. The results of the eight multiplex PCR using DNA purified from strains correlated 100% with the Quellung reaction applied in the live cells. Moreover, the same methodology, now applied to the DNA extracted from the 25 CSF samples which had viable strains, was able to genotype 24 samples that correlated with the results of the PCR using purified DNA from isolated strain, and also with the Quellung results. Only one CSF sample was not possible to be identified in the eight multiplex PCR, with all master mixes tested. It is suggested that this negative result was due to the relatively high Ct value for the *lytA* gene in the qPCR (CT = 28), meaning a low quality of the bacterial DNA available.

Multiplex PCR with *lytA*-positive CSF samples

From January/2014 to September/2020, 873 clinical specimens were received for bacterial meningitis analyses, 149 samples (17.1%) were *S. pneumoniae* positive by detection of the *lytA* gene, whereas 129 were positive for *N. meningitidis* (14.8%) and 15 for *H. influenzae* (1.7%). The majority was negative for multiplex qPCR (66%). From 149 *lytA*-positive samples, 79 had enough material to perform the eight multiplex PCR. Detection of two bands in the agarose gel, one referring to the genotype group which varies in size (see Suppl 1) and the other to the positive control, the *cpsA* gene (160 bp), in one of the eight multiplex PCR, meant a positive result for genotyping identification. A total of 58 CSF samples (73.4%) had successfully the genotyping group identified; on the other hand, 21 (26.6%) were not genotyped. There was an association between the positive result for genotyping identification and the Ct value observed in the multiplex qPCR for bacterial meningitis, i.e., the Ct value for the *lytA* gene obtained with the CSF sample (Fig. 3). Frequently, genotyping identification was observed with CSF samples which presented statistically lower Ct value for *lytA* in the qPCR (mean 23.53 ± SE 0.614) and it was different from the unidentified samples, which presented higher Ct value (mean 34.67 ± SE 0.659), $p < 0.0001$. Moreover, when evaluating the type of extraction (silica column or heating), there was no association between the obtained Ct value for *lytA* and the success of genotyping identification (Fig. 4). Of the ungenotyped CSF samples (21), seven had only the *cpsA* gene amplified, so they presented just one band in all the eight multiplex PCR,



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[ ] ng/µL	6.4	5.2	0.3	18.7	6.8	0	77	8.5	2.1	121	20.4	88.7	102	32.1	47.9
total ng	608	494	28.5	935	340	0	3850	425	105	3020	510	2217	2552	802	1197

Fig. 2 Yield of the amplicons using templates with different quality for all master mixes kits tested. In yellow the quantification in nanograms (ng) in the agarose gel made by GelQuant Express—Image Analysis software. Lanes: 1 to 3—Invitrogen™ PCR Supermix Brasil (Thermo Fisher Scientific); 4 to 6—Platinum® Multiplex PCR Master Mix (Thermo Fisher Scientific); 7 to 9—2 × PCR SuperMix (Bio-Helix); 10 to 12—QIAGEN® Multiplex PCR Kit (Qiagen); 13 to 15—GoTaq® Hot Start Green Master Mix (Promega); 16—molecular weight. Templates features: A1—Purified DNA from a serotype 22F strain; A2—DNA extracted from CSF sample with high quality and identified as the presence of serotype 8 strain; A3—DNA extracted from CSF sample with low quality and identified as the presence of serotype 22F strain (same CSF sample from which the strain presented in lanes A1 was isolated). The arrow indicates the 160 bp *cpsA* gene; The dotted arrow indicates unspecific bands. The table below the figure represents: [ ] ng/µL—concentration in ng of the amplified band correspondent to the genotyping, excluding the band referring to the *cpsA* gene; total ng—the total amount of produced DNA in ng contained in the total reaction volume correspondent only to the genotyping band

and 14 had no band at all. This result is also associated with the Ct value of the multiplex qPCR for the *lytA* gene (chi-square $p = 0.0005$); samples with higher Ct values presented 0 or 1 band in the gel (Fig. 5).

Furthermore, the genotype group identification from CSF samples presented different results according to the master mix applied in the eight multiplex PCR. Due to the availability and low cost of some master mixes kits, CSF samples were firstly tested with Platinum® Multiplex PCR Master Mix or Invitrogen™ PCR Supermix Brasil (both from Thermo Fisher Scientific), and in the absence of positive results, they were tested with QIAGEN® Multiplex PCR Kit (Qiagen). If positive results were not achieved, the CSF sample was submitted to the eight multiplex reactions with 2 × PCR SuperMix (Bio-Helix) and GoTaq® Hot Start Green Master Mix (Promega), which presented great results in the gel quantification shown previously in Fig. 2. Figure 6 presents, as expected, differences according to the master mix applied in the ability to identify the genotype group of *S. pneumoniae* in CSF samples. QIAGEN® Multiplex PCR Kit was the best master mix to identify the pneumococcus genotype. It presented 65.6% of positive results (42 samples) from 64 CSF samples tested with this master mix. Additionally, 22 CSF samples that were not identified by QIAGEN® Multiplex PCR Kit were tested with other master mixes available (2 × PCR SuperMix and GoTaq® Hot Start Green Master Mix), and only one was possible to identify the genotype group with 2 × PCR SuperMix (Bio-Helix). Moreover, the comparison between the success of genotype identification and the different master mixes used in this study showed statistical significance for QIAGEN® Multiplex PCR Kit (chi-square and Fisher tests $p < 0.0005$).

With all these findings, it was possible to evaluate a model of prediction based on the obtained Ct value for the *lytA* gene in the qPCR, and the probability to identify successfully the genotype (Fig. 7). Other variables were tested, but none showed any association. Also, a ROC curve was designed (Fig. 8). Both results showed that a Ct value around 30 has the chance to get a true positive result of about 75%; for a Ct value of 33, the chance for identification is 38%, and higher than a Ct value of 35, this chance drops to 12.5%.

Finally, with all genotyping results, it was possible to evaluate the pneumococcus genogroups that caused meningitis in the region in a retrospective study (Fig. 9). From 2014 to 2020, the genotype 19A was the most prevalent in the period (12%), followed by genotype 3 and 12F/12A/44/46 group (10% for each group). Despite the isolated strain, which had the CSF sample negative in the eight multiplex PCR, being identified by the Quellung reaction as serotype 23A, it was counted as not a genotyped sample. Moreover, when analyzing the period when CSF samples were cultured (2017–2020), it was possible to observe that from 377 CSF samples only 25 *S. pneumoniae* strains were isolated in culture and the Quellung reaction could be performed with the viable strains. However, the qPCR identified 69 CSF samples positive for *S. pneumoniae* by the *lytA* gene detection. In the absence of the eight multiplex PCR, it would be possible to identify the serotype for only 25 isolated strains (25/69; 36.2%). Nonetheless, this study was able to identify 42 genotypes from 69 CSF *lytA*-positive samples, representing 60.9% of the *lytA*-positive samples in the period 2017–2020.

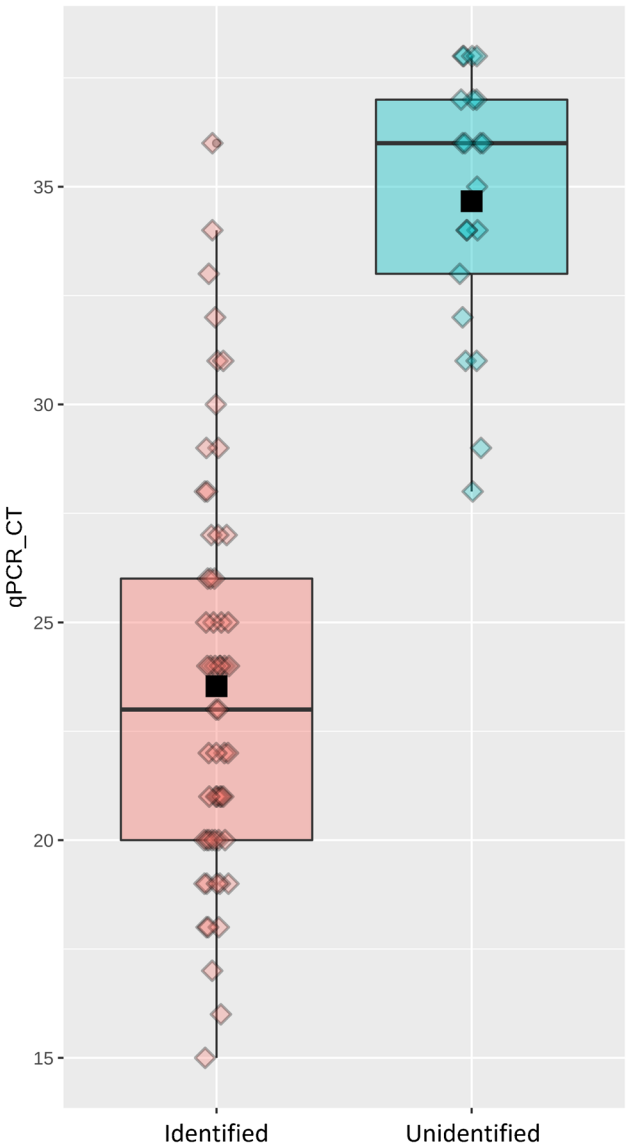


Fig. 3 Cycle threshold for the *lytA* gene of the bacterial meningitis multiplex qPCR (qPCR_CT) versus genotyping identification using the eight multiplex PCR with CSF samples. Each diamond represents a sample. The black bar represents the median and the black square represents the mean

Discussion

The culture of CSF samples is the gold standard for bacterial meningitis. However, many reasons can contribute to false negative results. PCR-based assays can overcome these issues and can be an auxiliary tool that complements the main technique. As observed in this study, in which 72.6% of CSF samples were culture negative, at least 33% of them were positive in the qPCR for the main three bacteria that cause meningitis. This positivity of the CSF samples in the qPCR, in the region of this study, was similar to our previous finding (33.8%) that evaluated a longer period [15]. Also, 41.7% of the cultured samples were positive for one microorganism which was not contemplated in the qPCR. The high percentage of culture-positive CSF for other microorganisms shows the importance of the gold standard methodology to diagnose other bacterial meningitis, also the results of qPCR-positive but culture-negative show the complementarity between the assays. Therefore, these findings indicate the importance of both methodologies and they should be performed together routinely to promote more consistency in the results. Moreover, a greater recovery percentage of strains was observed for *S. pneumoniae* (52%); thus, it is possible to infer that this bacterium was less sensible to hostile conditions in which the CSF samples were submitted before it was cultured in our laboratory. When the subject is the surveillance of the serotypes or serogroups that cause bacterial meningitis, PCR-based methodologies can be a better tool, because they can identify even in the absence of the isolated strain. According to this study, the eight multiplex PCR can be applied to CSF samples successfully, and the chance to get the pneumococcus strain identified is almost 100% if the Ct value for the *lytA* gene in the qPCR is less than 25.

The CDC protocol, which was the basis of this study, applied the QIAGEN® Multiplex PCR Kit for DNA extracted from isolated strains. Also, they applied an annealing temperature of 54 °C for the eight multiplex PCR. Here, we tested others master mixes kits to identify if any enzyme

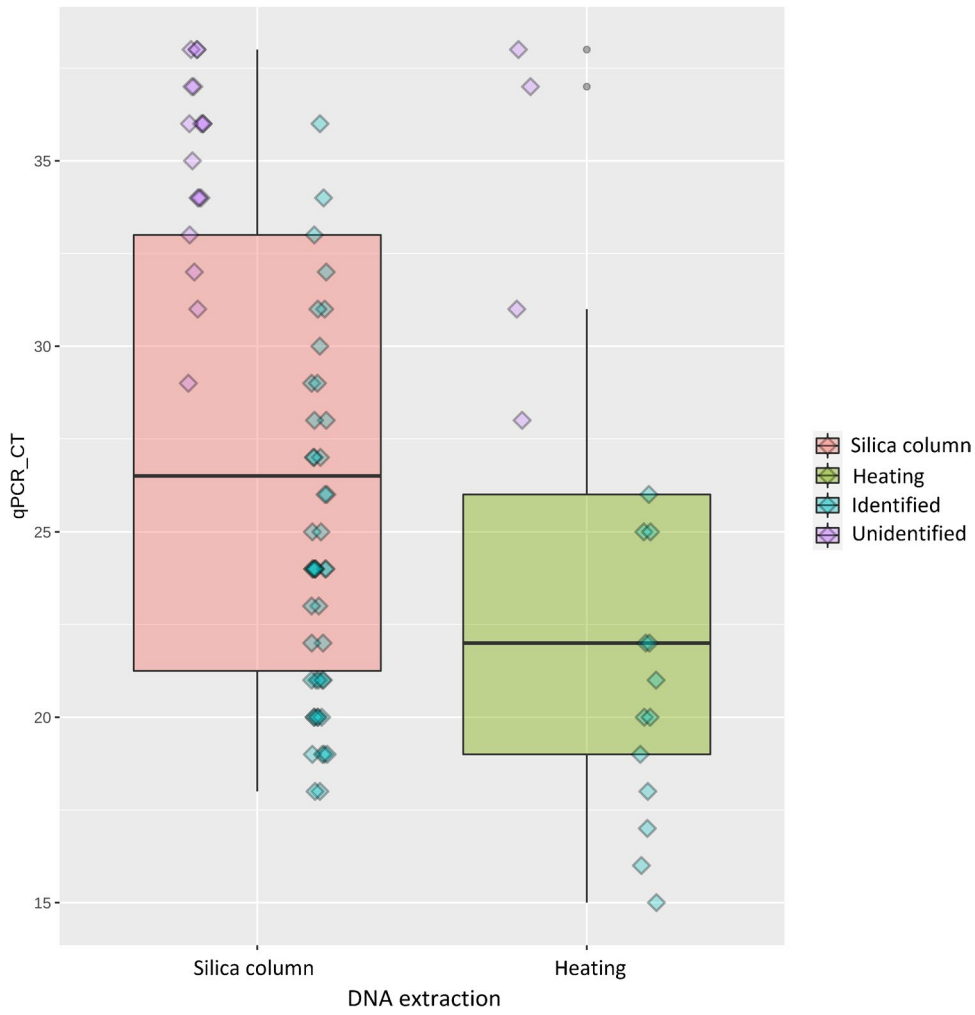


Fig. 4 Cycle threshold for the *lytA* gene of the bacterial meningitis multiplex qPCR (qPCR_CT) versus the type of DNA extraction (silica column or heating) and the result of genotyping identification using the eight multiplex PCR. The black bar represents the median

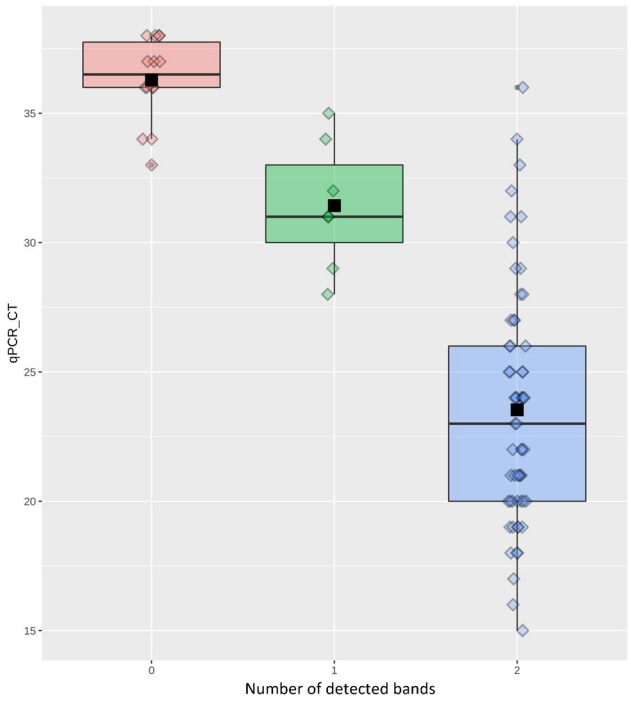


Fig. 5 Correlation of cycle threshold for the *lytA* gene of the bacterial meningitis multiplex qPCR (qPCR_CT) and the number of bands detected in the agarose gel after the eight multiplex PCR using CSF samples: 0 — no bands were detected; 1 — only one band was detected and it corresponds to the *cpsA* gene, presented in all *S. pneumoniae* strains, used here as positive control; 2 — two bands were detected, referring to the *cpsA* gene and the gene which represents the genotype evaluated. Only two bands were considered positive for genotyping identification. The black bar represents the median and the black square represents the mean

could be applied and also if the protocol is the same for CSF samples. Other master mixes were applied according to the availability in our laboratory and the variable costs. It was an attempt to reduce the cost of this methodology. Platinum® Multiplex PCR Master Mix and QIAGEN® Multiplex PCR Kit were designed for multiplex reactions. They presented similar costs for this study (US$ 3.8 and 3.2 per reaction). On the other hand, they presented different sensitivity, as the results presented here. For QIAGEN® Multiplex PCR Kit, CDC has standardized reactions using half of the master mix, thus decreasing the cost in this study to US$ 1.6. Other master mix kits tested here had lower costs (US$ 0.6 or 0.3 per reaction), 2.5 or 5 times less than QIAGEN® Multiplex PCR Kit. As observed here, to reduce the cost of this assay, other enzymes could be applied, but maybe, it will promote the increase of negative results (absence of genotyping). When different master mixes were applied the annealing temperature was the same; however, when the QIAGEN® Multiplex PCR Kit was applied, unspecific reactions were observed and multiple bands were detected when using DNA extracted from CSF samples (data not shown). For this reason, some modifications in the eight multiplex PCR protocols were necessary to deal with this issue; thus, different annealing temperatures were standardized for each one of the eight multiplex PCR. This result allied to the result that QIAGEN® Multiplex PCR Kit was the best master mix to identify the pneumococcus genotype, with 65.6% of positive results, suggesting that this master mix is more sensitive than the others tested here. Thus, the success of pneumococcus genotype identification will also depend on the master mix applied in the study. Furthermore, despite better and new PCR-based methodologies available, our work applied conventional PCR because it is cheaper and easier to be applied all over the world, especially in low-income countries, where pneumococcus is prevalent.

When comparing both methodologies, the Quellung reaction performed with isolated strains and the eight multiplex PCR performed with the CSF samples in which these strains were isolated, only one CSF sample in 26 was not genotyped. All 25 CSF samples presented the same result as the gold standard Quellung reaction. Regardless of the small number of isolates, this evidence still is valid to endorse the use of the eight multiplex PCR to genotype *S. pneumoniae* in CSF samples, especially in the absence of isolated strains, as a complementary technique. Thus, our results show that the eight multiplex PCR developed by the CDC for isolated pneumococci strains can be successfully applied to CSF samples, with protocol modifications presented here.

Based on the methodology of eight multiplex PCR, we were able to perform a retrospective study using all *lytA*-positive CSF samples, which had great volumes and were received between 2014 and 2020. We obtained 73.4% of all CSF samples successfully genotyped; 26.6% of CSF samples were not genotyped. The great percentage of positives also indicates this methodology should be applied as a routine of surveillance since most of them would have remained unidentified if the CSF samples had not been tested. For seven (from 21) not genotyped samples, a single band was observed in all eight multiplex PCR, representing the *cpsA* gene. This observation could be explained by other genogroups not included in the eight multiplex PCR tested in this study; however, the hypothesis of the low quality of the sample, as observed for samples with a higher Ct value for the *lytA* gene, is more plausible. Furthermore, one of these seven samples had a pneumococcus strain isolated by culture, thus the serotype was confirmed by Quellung reaction, and this serotype is included in the eight multiplex PCR. This information reinforces that the quality and concentration of the bacterial DNA presented in the CSF sample should be high. Carvalho and coworkers [6] described that despite the *cpsA* gene being highly conserved among pneumococcal strains, they found 1 to 2% of the PCR-serotypeable isolates negative for this control.

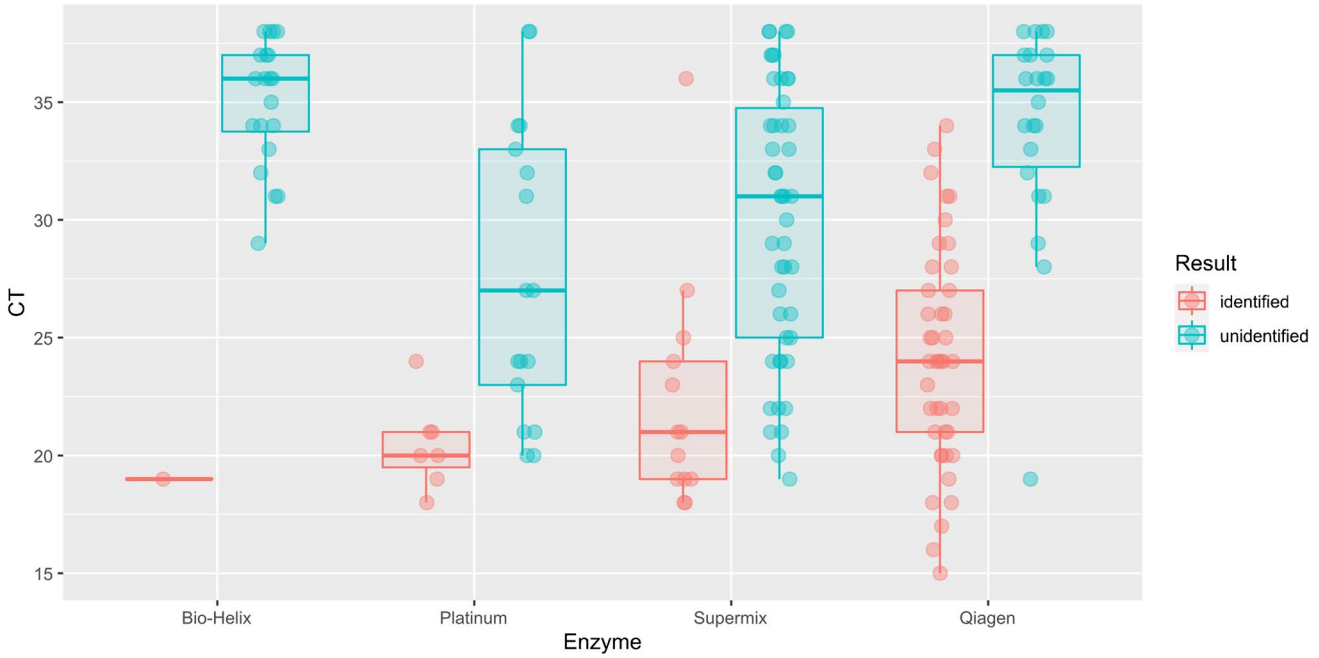


Fig. 6 Genotype identification after the eight multiplex PCR using CSF samples as templates and 4 different master mixes versus the cycle threshold (CT) for the *lytA* gene from the bacterial meningitis multiplex qPCR. Master mixes applied: 2×PCR SuperMix Bio-Helix (Bio-Helix); Platinum® Multiplex PCR Master Mix (Platinum); Invitrogen™ PCR Supermix Brasil (Supermix); QIAGEN® Multiplex PCR Kit (Qiagen). CSF samples with the genotype identified by one of the eight multiplex PCR are represented in red and unidentified in blue

This could be the explanation for the 14 not genotyped samples. Nevertheless, all samples presented the Ct value of > 33 for the *lytA* gene, indicating low bacterial DNA concentration. Moreover, according to the model of prediction observed in our study, CSF samples with this Ct value has also a small chance to be identified (38%). Therefore, these results indicate that, like all methodologies, this PCR-based technique applied to CSF samples has its limitation, but it should not be discredited as a potent tool to genotype *S. pneumoniae*, mainly in the absence of isolated strains.

Lastly, the results of pneumococcus genotyping based on the eight multiplex PCR applied here with CSF samples are unprecedented, since the region of this study, which comprises

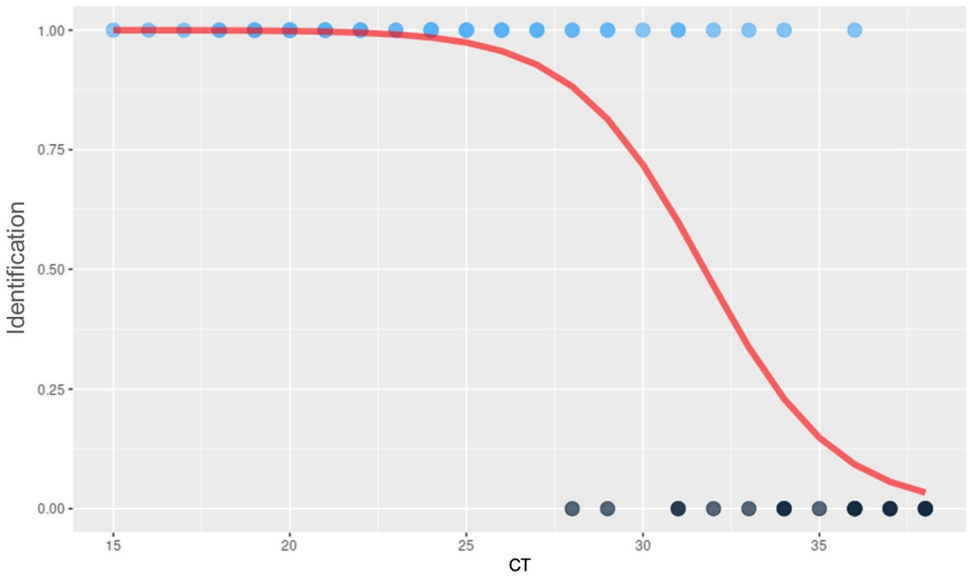


Fig. 7 Model of prediction of the genotyping result using the eight multiplex PCR with CSF samples based on the obtained cycle threshold for the *lytA* gene of the bacterial meningitis multiplex qPCR (CT). Identification means the probability of successfully genotyped samples using this methodology, of which 0 is 0% identified, 0.50 is 50% identified, and 1.0 is 100% identified. The red line shows the decrease of identification probability as the Ct value increase

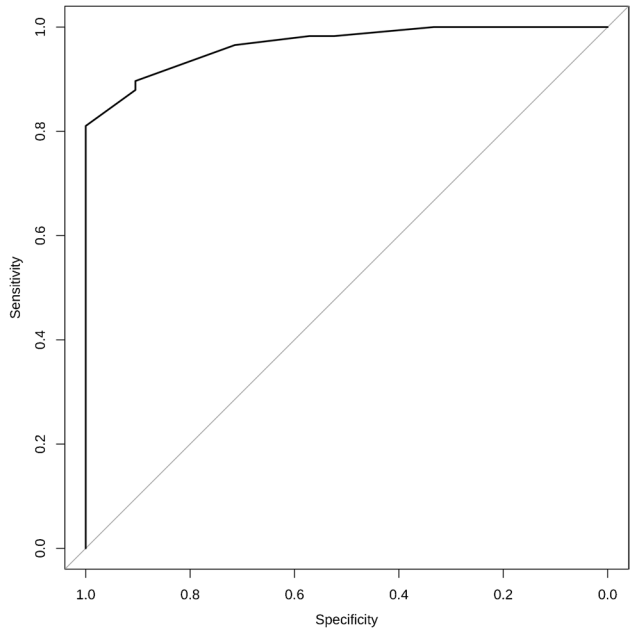


Fig. 8 Receiver operating characteristic (ROC) curve

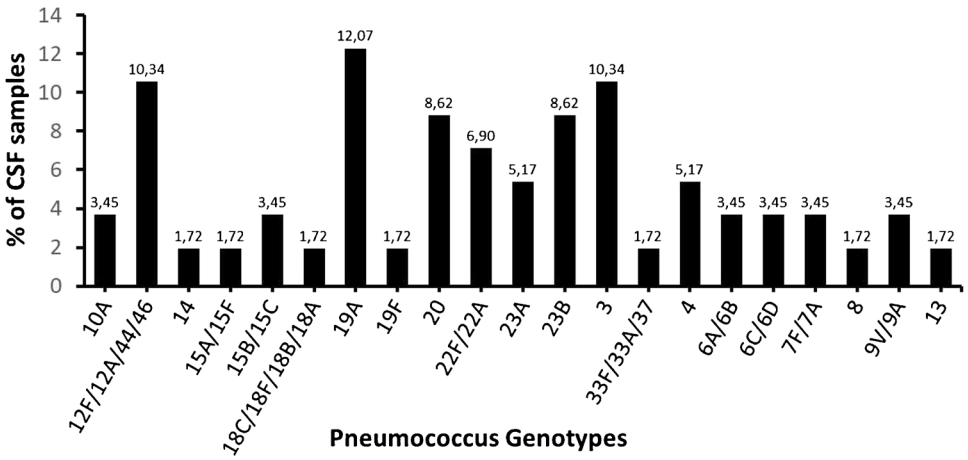


Fig. 9 Serotype profile of *S. pneumoniae*, in the period of 2014–2020, identified by the eight multiplex PCR with CSF samples

more than 2.5 million people, has not the observance to provide isolated strains for further epidemiological investigation, as recommended by the country's epidemiological surveillance standards. Serotype 19A was the prevalent strain, as observed in many studies in Brazil, including invasive pneumococcal diseases and nasopharyngeal carriage [16–21], due to the use of the 10-valent vaccine (PCV10) in the Brazilian immunization program that does not include this serotype, and for this reason, there is an increase in the non-vaccine serotypes. All CSF samples which presented the 19A genotype were also identified as multidrug-resistant in our previous work [22]. There was an increase worldwide in the incidence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A. This was especially observed for the serotype 19A clonal complex CC320 [19, 23–26]. The second most common serotype in this study was serotype 3. It is also not included in the PCV10 provided in Brazil. The prevalence of serotypes 19A and 3 was similar to the study of Quesada and coworkers in Brazil [21]. However, they found serotype 6C as one of the top three prevalent, and in our work, this was not observed. Curiously, Quesada and coworkers found that serotype 3 is also the prevalent serotype causing meningitis in older than 5 years of age in sites where PCV13 was used, a vaccine in which serotype 3 is included [21].

Based on our findings, we concluded that the use of CSF samples to genotype the *S. pneumoniae* by the eight multiplex PCR is feasible and reliable and could be applied in the routine diagnosis of bacterial meningitis, especially in the absence of the isolated strain, helping in the surveillance of pneumococci serotypes causing meningitis. Despite the fact this assay may not detect the serotype in CSF samples with low DNA quality, it is still worth the attempt to obtain missing information about the strain. The results obtained here evidence the importance of the implantation of the eight multiplex PCR for genotyping prediction applied to CSF samples in the routine of epidemiological surveillance, which is extremely important to verify if the serotype replacement phenomenon is occurring and to better establish immunization policies.

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Author contribution Conception and design of the study: IBC; Material preparation and data collection: MBS, DRC, DAM; Formal analysis and investigation: MBS, EC, MCCN, IBC; Resources: IBC, AMSC, VSMGD; Funding acquisition: IBC, AMSC, VSMGD; Writing—original draft preparation: IBC, EC, MCCN; Writing—review and editing: EC, AMSC, VSMGD, IBC; Supervision: IBC. All authors have read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval The Institutional Ethical Committee from Adolfo Lutz Institute has approved this work by CAAE nº 80295817.7.0000.0059 and waived the informed consent from patients for this study and forthcoming publication since clinical specimens reported here were collected for diagnostical purposes and data were analyzed anonymously.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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