The Journal of Infectious Diseases

SUPPLEMENT ARTICLE

Triplex Direct Quantitative Polymerase Chain Reaction for the Identification of *Streptococcus pneumoniae* Serotypes

Mahamoudou Ouattara,[1] Mamadou Tamboura,[2] Dinanibè Kambiré,[2] Kim Anh Lê,[3] Thanh Van Phan,[4] Srinivasan Velusamy,[1] Hien Anh Nguyen,[3] Dai Vo Thi Trang,[4] Fernanda C. Lessa,[1] Makiko Iijima,[5] Dac Trung Nguyen,[3] Stephanie B. Schwartz,[1] Lesley McGee,[1] Rasmata Ouédraogo Traoré,[2] and Bernard Beall[1]

[1]Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA, [2]Centre Hospitalier Universitaire Pédiatrique Charles de Gaulle, Ouagadougou, Burkina Faso, [3]National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, [4]Pasteur Institute of Ho Chi Minh City, Ho Chi Minh City, Vietnam, and [5]World Health Organization Representative Office for Vietnam, Expanded Programme on Immunization, Hanoi, Vietnam

The quantitative polymerase chain reaction (qPCR) method presented in this study allows the identification of pneumococcal capsular serotypes in cerebrospinal fluid without first performing DNA extraction. This testing approach, which saves time and resources, demonstrated similar sensitivity and a high level of agreement between cycle threshold values when it was compared side-by-side with the standard qPCR method with extracted DNA.

Keywords. *Streptococcus pneumoniae*; direct real-time PCR; pneumococcal serotype.

Streptococcus pneumoniae is a leading bacterial etiology of a wide range of infections, including community-acquired pneumonia, meningitis, otitis media, and sepsis [1]. There are now 100 recognized *S. pneumoniae* serotypes whose distribution varies both temporally and geographically [2]. Quantitative polymerase chain reaction (qPCR) methods for the identification of *S. pneumoniae* serotypes using DNA extracted from clinical specimens or pneumococcal isolates have been described previously [3–5]. It is a laborious process, and the DNA extraction step can constitute a source of cross-contamination and a bottleneck in high-throughput laboratories. To alleviate some of the burden associated with the above-mentioned traditional qPCR, we validated a direct qPCR method that allows the identification of *S. pneumoniae* serotypes directly from cerebrospinal fluid (CSF) specimens positive for *S. pneumoniae* by *lytA* qPCR without first extracting DNA, thereby reducing processing time, cost, labor, and risk of cross-contamination.

MATERIALS AND METHODS

The triplex direct qPCR method was validated using the same schemes and the same oligonucleotides as described by Pimenta et al [3], with the exception that the final concentrations of the oligonucleotides were optimized in the direct qPCR (Table 1).It consists of 7 sequential triplex reactions (21 assays) that identify 37 pneumococcal capsular serotypes as 11 individual serotypes plus 10 small serogroups. Reaction mixtures were prepared in a final volume of 25 µL, including variable volumes of each primer and probe to reach the desired final concentration, 2 µL of CSF as DNA template, 12.5 µL of mastermix (PerfeCTa MultiPlex qPCR ToughMix, QuantaBio), and PCR-grade water (Supplementary Materials). The thermal profile condition for the qPCR runs was 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each run included appropriate positive control DNA and no template negative controls (NTCs). A reaction was considered valid if all NTCs were negative and control DNA was positive.

The lower limits of detection (LLD) for the traditional and direct PCR methods in this study were determined as described previously [3, 6]. In brief, bacterial suspensions of target serotypes were prepared in 0.85% saline buffer to a turbidity reading of approximately 0.5 McFarland standard. These suspensions were further serially 10-fold (10^{-1} to 10^{-7}) diluted in 0.85% saline buffer and in CSF that had been previously tested and known to be PCR negative. DNA was extracted from 200 µL of serial dilution suspensions as previously described [7]. The DNA concentrations were measured using a NanoDrop instrument and plotted against cycle threshold (Ct) values. The concentration that yielded a Ct of 35 was considered the LLD.

RESULTS

The LLD of the individual serotypes for the direct qPCR, determined as described above, varied between 7 and 16 genome equivalents per reaction (3.5–8 genome equivalents/µL CSF), which were comparable to the LLD obtained for the traditional qPCR (between ~7.5 and ~15 cell genome equivalents per reaction [3]).

The direct qPCR and the qPCR method with extracted DNA were compared concurrently, using the same set of 120 CSF specimens that had tested positive for the *S. pneumoniae* gene target,

Correspondence: Mahamoudou Ouattara, PhD, Division of Bacterial Diseases, Centers for Disease Control and Prevention, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Atlanta, GA 30329 (mouattara1@cdc.gov).

The Journal of Infectious Diseases® 2021;224(S3):S204–8
Published by Oxford University Press for the Infectious Diseases Society of America 2021. This work is written by (a) US Government employee(s) and is in the public domain in the US.
DOI: 10.1093/infdis/jiab056

Table 1. Primer and Probe Sequences and Concentrations Used in the Study

Primer/Probe ID [3]	Sequence (5′ – 3′) [3]	Probe Dye [3]	Probe Special Chemistry [3]	Quencher (3′) [3]	Traditional PCR, nM [3]	Direct PCR, nM
1-F	TTTCATCCCTATGTGTGGTATAG				300	600
1-R	GCTTTAGAAGGTAGAGTTAACAAC				300	600
1-Probe	TGCCAAAGCCAGCCAT	FAM	LNA[a]	BHQ1	100	200
2-F	TGTTATCCCATATAAGAACCGAGTGT				300	600
2-R	AAAATTACCCCAAAAGCTATCCAA				300	600
2-Probe	TTGCAATT"T"CAATTTTTTTGCCCCAATCTC	FAM	"T"[b] = BHQ1	BHQ1	200	400
3-F	CCACTAAAGCTTTGGCAAAAGAAA				300	600
3-R	CCCGAACGTAAAGCTTCTTCA				300	600
3-Probe	TTGTAGACCGCCCCACAA"T"TCATTTTGT	HEX	"T"[b] = BHQ1	BHQ1	200	400
4F	GCTTCTGCTGTAACTGTTGTGC				300	600
4-R	CACCACCATAGTAACCAAAGTTCC				300	600
4-Probe	TTCCACAAAAGAAGAGCCTACAGGTAACCCCA	ROX		BHQ2	100	200
5-F	CATGATTTATGCCCTCTTGCAA				300	600
5-R	GACAGTATAAGAAAAAGCAAGGGCTAA				300	600
5-Probe	TCTTCTTCTCA"T"CGTTTCCGCATGCTTTT	HEX	"T"[b] = BHQ1	BHQ1	200	400
6A/6B/6C/6D-F	GTTTGCACTAGAGTATGGGAAGG				200	400
6A/6B/6C/6D-R	TAGCCTTTCTGAAAACATTTAGCG				200	400
6A/6B/6C/6D-Probe	TGTTCTGCCC"T"GAGCAACTGGTCTTGTATC	FAM	"T"[b] = BHQ1	BHQ1	200	400
6C/6D-F	TTGGGATGATTGGTCGTATTAG				200	400
6C/6D-R	CTCTTCAATTAGTTCTTCAGTTCG				200	400
6C/6D-Probe	CCACGCAATTCGCCATC	FAM	LNA[a]	BHQ1	100	200
7F/7A-F	ATGAAGGCTTTGGTTTGACAGG				200	400
7F/7A-R	ATTCTCGCCATCAATTGCATATTC				200	400
7F/7A-Probe	ACACCACTATAGGCTGTTGAGACTAACGCACA	ROX		BHQ2	100	200
9V/9A-F	AGGTATCCTATATACTGCTTTAGG				300	600
9V/9A-R	CGAATCTGCCAATATCTGAAAG				300	600
9V/9A-Probe	ACACATTGACAACCGCT	HEX	LNA[a]	BHQ1	100	200
11A/11D-F	AAATGGTTTGGATATGGTTTGTTTGG				300	600
11A/11D-R	AAATGGTTTGGATATGGTTTGTTTGG				300	600
11A/11D-Probe	ATTCCAACTTCTCCCAATTTCTGCCACGG	ROX		BHQ2	100	200
12F/12A/12B/44/46-F	GCACCCACGGGTAAATATTCTAC				300	600
12F/12A/12B/44/46-R	CAACTAAGAACCAAGGATCCACAG				300	600
12F/12A/12B/44/46-Probe	TGCCCACCAACACCAGGTCCAGGT	ROX		BHQ2	200	400
14-F	AGAGTGTATGAGGAATCC				300	600
14-R	ATATATCTACTGTAGAGGGAAT				300	600
14-Probe	CGCCAAGTAACA"T"TTCCATTCCATT	FAM	"T"[b] = BHQ1	BHQ1	100	200
15A/15F-F	AATTGCCTATAAACTCATTGAGATAG				200	400
15A/15F-R	CCATAGGAAGGAAATAGTATTTGTTC				200	400
15A/15F-Probe	CCCGCAAACTCTGTCCT	FAM	LNA[a]	BHQ1	100	200
16F-F	TAATGTTATGACCTTGGTAATCTTCCC				300	600

Table 1. Continued

Primer/Probe ID [3]	Sequence (5′ – 3′) [3]	Probe Dye [3]	Probe Special Chemistry [3]	Quencher (3′) [3]	Traditional PCR, nM [3]	Direct PCR, nM
16F-R	TCCCAAAGGATAATCAATAACTTTTAGAAG				300	600
16F-Probe	AGCCATAAGTCT"T"CCAAATGCTTAACCGCT	HEX	"T"[b] = BHQ1	BHQ1	100	200
18C/18A/18B//18F-F	TCGATGGCTAGAACAGATTTATGG				200	400
18C/18A/18B/18F-R	CCATTGTCCCTGTAAGACCATTG				200	400
18C/18A/18B/18F-Probe	AGGGAGTTGAATCAACCTATAATTTCGCCCC	HEX		BHQ1	100	200
19A-F	CGCCTAGTCTAAATACCA				200	400
19A-R	GAGGTCAACTATAATAGTAAGAG				200	400
19A-Probe	TATCAATGAGCCGATCCGTCACTT	FAM		BHQ1	100	200
19F-F	TGAGGTTAAGATTGCTGATCG				300	600
19F-R	CACGAATGAGAACTCGAATAAAAG				300	600
19F-Probe	CGCACTGTCAATTCACCTTC	ROX	LNA[a]	BHQ2	100	200
22F/22A-F	TCTATTAAATAACCCATTGGAATTGAAACG				200	400
22F/22A-R	TCGCAATTGAAGACCACATAAACTG				200	400
22F/22A-Probe	TCCGTAAT"T"CGCTTATGGGCACATTCTCCA	HEX	"T"[b] = BHQ1	BHQ1	200	400
23A-F	CTCCCCTCCATTACCCATTTGG				200	400
23A-R	TGAAGAAAGTGCTGTTTGTGAACC				200	400
23A-Probe	AGCTAGAAC"T"CCCACACTCCCTACTCCCA	ROX	"T"[b] = BHQ2	BHQ2	100	200
23F-F	GACAGCAACGACAATAGTCATCTC				300	600
23F-R	TCCATCCCAACCTAACACACTTC				300	600
23F-Probe	ATTGTGTCCA"T"AACCCTTCGTCGTATTTCCAAAG	ROX	"T"[b] = BHQ2	BHQ2	200	400
33F/33A/37-F	GGAACTGGTTCAGCAACTATACG				200	400
33F/33A/37-R	GGTTCTAAGACCGTCTGAAATAC				200	400
33F/33A/37-Probe	CCCCAAATAGGAC"T"TTTCTGCCATGCCAAA	HEX	"T"[b] = BHQ1	BHQ1	200	400

[a]Locked nucleic acid nucleotides are underlined.

[b]"T" indicates a black hole quencher placed internally on the thymidine base.

lytA [6], at the national reference laboratories for meningitis surveillance in Burkina Faso (n = 85) and Vietnam (n = 35). For traditional qPCR, DNA was extracted from a 200-µL aliquot of each CSF specimen as described above. To measure the agreement between the 2 methods, CSF specimens and their corresponding extracted DNA were tested concomitantly on the same plate by the direct and the traditional methods, respectively. The Ct difference (ΔCt) between the direct and the traditional PCR ($Ct_{Direct} - Ct_{DNA}$) with extracted DNA was plotted as a function of the average Ct values of the 2 methods [8]. The upper and lower limits of agreement between the 2 methods were calculated as the mean ΔCt ± 2 standard deviations (Figure 1A). If the 2 methods agree, we expect at least 95% of the ΔCt to fall within the limits of agreement. In this plot, only 2 data points (~1.7 %) were outside the limits of agreement, demonstrating a very good strength of agreement between the direct and the traditional PCR. Specimens that were found negative for the 37 serotypes (n = 29) by direct PCR were also negative when the PCR was conducted on their extracted DNA. There was no discrepancy among the serotypes identified by the 2 methods. Because this was a prospective study (the serotypes were not known prior to the study) in 2 specific regions, serotypes/serogroups 4, 19A, 23A, 6C/6D, 7F/7A, 22F/22A, and 33F/33A/37 were not detected in the CSF tested.

A corrected Bland–Altman plot (Krouwer plot) suggests that if a new method is being compared to a reference method, one should plot the difference against the values of the reference method rather than the average values of both methods [9]. When the traditional qPCR was considered as the reference method and ΔCt was plotted as a function of Ct_{DNA}, the observed agreement between the 2 methods was unchanged (Figure 1B). In addition to the high level of agreement, there was a strong positive correlation between Ct values generated by the 2 methods (Pearson correlation coefficient: $r = 0.86$; $P < .00001$), which indicates that high Ct values of one assay correspond to high Ct values of the other and vice versa.

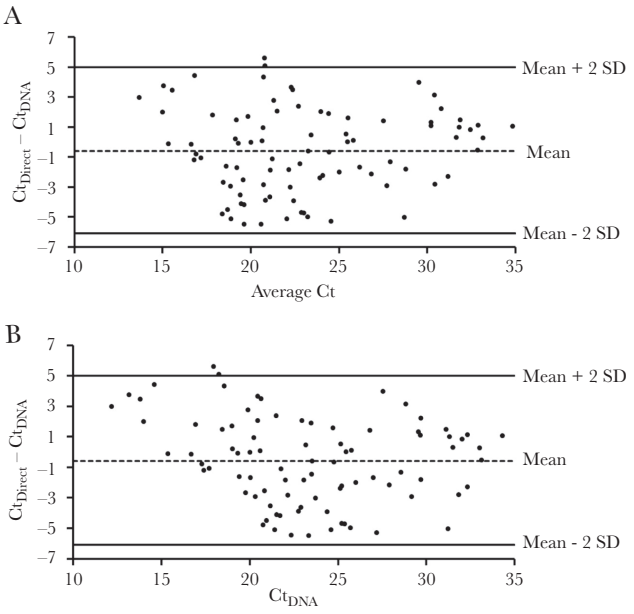

Figure 1. Bland–Altman plot (*A*) and Krouwer plot (*B*) of the cycle threshold differences (ΔCt) between the direct real-time polymerase chain reaction (PCR) method and the traditional real-time PCR method. The upper and lower limits of agreement are indicated by the upper and lower solid lines (SD = standard deviation of ΔCt). The dashed line indicates the mean ΔCt and the dots represent ΔCt values. These data were generated using 120 clinical cerebrospinal fluid samples.

DISCUSSION

Identification of pneumococcal capsular serotypes is very important for monitoring the temporal and geographical distribution of disease-causing serotypes and for measuring pneumococcal conjugate vaccine impact. Molecular methods such as qPCR that allow the identification of serotypes most frequently associated with disease in humans are widely used and are perpetually being optimized. Recently, an expanded sequential PCR scheme consisting of 14 quadriplex reactions that identify 64 individual serotypes/serogroups, antibiotic resistance, and pili genes has been published [10]. The direct qPCR described here contributes to that optimization effort by considerably reducing labor and processing time and, most importantly, saving valuable specimen volumes. In addition, the sensitivity and the specificity of the direct qPCR are comparable to those of traditional qPCR for the identification of *S. pneumoniae* serotypes. With the recent validation of a triplex direct qPCR for the detection of bacterial species in CSF, including *S. pneumoniae* [6], it is now possible to rapidly and accurately detect this important pathogen and its capsular serotypes directly in CSF without first performing DNA extraction. We are planning to expand the direct qPCR to the new quadruplex assay that includes a larger number of serotypes.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the official position of the United States Centers for Disease Control and Prevention (CDC).

Supplement sponsorship. This supplement is sponsored by the World Health Organization and the U. S. Centers for Disease Control and Prevention.

Financial support. This work was supported by the CDC.

Potential conflicts of interest. All authors: No reported conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Weiser JN, Ferreira DM, Paton JC. *Streptococcus pneumoniae*: transmission, colonization and invasion. Nat Rev Microbiol **2018**; 16:355–67.
2. Ganaie F, Saad JS, McGee L, et al. A new pneumococcal capsule type, 10D, is the 100th serotype and has a large *cps* fragment from an oral *Streptococcus*. mBio **2020**; 11:e00937-20.
3. Pimenta FC, Roundtree A, Soysal A, et al. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol **2013**; 51:647–52.
4. Sakai F, Chochua S, Satzke C, et al. Single-plex quantitative assays for the detection and quantification of most pneumococcal serotypes. PLoS One **2015**; 10:e0121064.
5. Tarragó D, Fenoll A, Sánchez-Tatay D, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin Microbiol Infect **2008**; 14:828–34.
6. Ouattara M, Whaley MJ, Jenkins LT, et al. Triplex real-time PCR assay for the detection of *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* directly from clinical specimens without extraction of DNA. Diagn Microbiol Infect Dis **2019**; 93:188–90.
7. Carvalho Mda G, Tondella ML, McCaustland K, et al. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol **2007**; 45:2460–6.
8. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet **1986**; 1:307–10.
9. Krouwer JS. Why Bland-Altman plots should use X, not (Y+X)/2 when X is a reference method. Stat Med **2008**; 27:778–80.
10. Velusamy S, Tran T, Mongkolrattanothai T, Walker H, McGee L, Beall B. Expanded sequential quadriplex real-time polymerase chain reaction (PCR) for identifying pneumococcal serotypes, penicillin susceptibility, and resistance markers. Diagn Microbiol Infect Dis **2020**; 97:115037.