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Triplex real-time PCR assay for the detection of *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* directly from clinical specimens without extraction of DNA

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ABSTRACT

This study presents a triplex real-time PCR assay that allows for the direct detection of *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* in one reaction without DNA extraction, with similar sensitivity and specificity to singleplex assays. This approach saves time, specimen volume and reagents while achieving a higher testing throughput.

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Streptococcus pneumoniae (Sp), *Neisseria meningitidis* (Nm), and *Haemophilus influenzae* (Hi) are three major pathogens frequently associated with bacterial meningitis worldwide (Coureuil et al., 2017). Many public health laboratories employ molecular methods for detection and characterization of these fastidious pathogens since obtaining viable organisms for culture from remote areas can be challenging. Both singleplex and multiplex real-time PCR assays are available for the detection of meningitis pathogens using DNA extracted from clinical specimens (Carvalho Mda et al., 2007; Dolan Thomas et al., 2011; Wang et al., 2011, 2012). Recently, three singleplex direct real-time PCR assays were validated for the detection of Sp, Nm, and Hi from cerebrospinal fluid (CSF) specimens, without performing DNA extraction (Vuong et al., 2016). The study showed that the direct real-time PCR method using singleplex assays detected meningitis pathogens with similar or better sensitivity than the traditional real-time PCR method that requires DNA extraction. In the present study, we validated a triplex direct real-time PCR assay that allows the direct detection of Sp, Nm and Hi in one reaction without DNA extraction.

The nucleotide sequences of the primers and probes used in this study (Table 1) were identical to those used in the singleplex direct real-time PCR method (Vuong et al., 2016), where FAM was the single fluorochrome used for all three singleplex direct real-time PCR assays for Sp, Nm and Hi. In order to combine the primers and probes targeting the three pathogens in a single triplex reaction, FAM, Cy5, and HEX were used for *sodC* (Nm target), and *lytA* (Sp target), and *hpd* (Hi target) specific probes, respectively. Each triplex or singleplex reaction was prepared in a final volume of 25 μl, including 1 μl of each primer and probe, 2 μl CSF as DNA template, 12.5 μl of mastermix (PerfeCta® MultiPlex qPCR ToughMix, QuantaBio) and sterile water. The thermal profile for the real-time PCR runs was 1 cycle of 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

The lower limits of detection (LLD) of the singleplex and the triplex assays in this study were determined in triplicate as described previously (Vuong et al., 2016). In brief, bacterial suspensions of target species (Sp, Nm or Hi) were prepared in Brain Heart Infusion broth (BHI) to a turbidity reading of approximately 0.2 OD_{600}. These suspensions

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Table 1
Sequences and concentrations of the primers and probes used in this study.

Target gene	Forward Primer (F)	Reverse Primer (R)	Probe (P)	Concentration of oligo (F/R/P) per reaction (nM)	Amplicon size
lytA [2]	ACG CAA TCT AGC AGA TGA AGC A	TCG TGC GTT TTA ATT CCA GCT	5TGC CGA AAA CGC "T" TGA TAC AGG GAG3 5′ Cy5; 3′ SpC6; "T" BHQ2	200/200/100	51 bp
sodC [4]	GCA CAC TTA GGT GAT TTA CCT GCA T	CCA CCC GTG TGG ATC ATA ATA GA	5CAT GAT GGC ACA GCA ACA AAT CCT GTT T3 5′ FAM; 3′ BHQ1	300/300/200	127 bp
hpd [3]	GGT TAA ATA TGC CGA TGG TGT TG	TGC ATC TTT ACG CAC GGT GTA	5TTG TGT ACA CTC CGT "T" GGT AAA AGA ACT TGC AC3 5′ Hex; 3′ SpC6; "T" BHQ1	100/900/300	151 bp

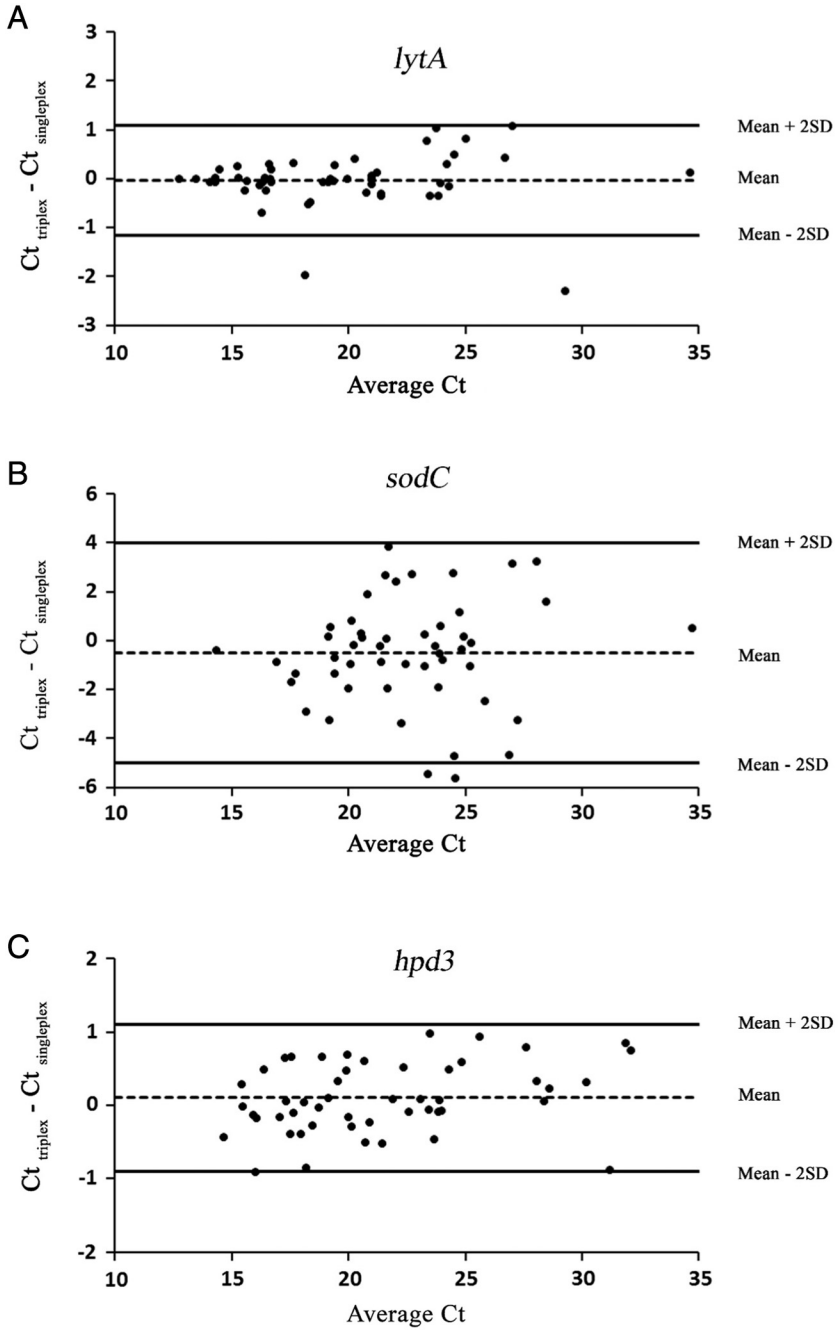

Fig. 1. Bland–Altman plots of the cycle threshold differences (ΔCt) between the triplex and the singleplex assays. The upper and lower limits of agreement for *lytA* (A), *sodC* (B) and *hpd* (C) are indicated by the upper and lower solid lines (SD = standard deviation of ΔCt). The dashed line indicates the mean ΔCt for each gene target.

were further serially diluted ten-fold (10^{-1} to 10^{-7}) in CSF that has been previously tested and known to be PCR negative and in BHI for enumeration of colony forming units per milliliter (CFU/ml). Bacterial concentrations (CFU/ml) were plotted against cycle threshold (Ct) values and the concentration that yielded a Ct of 35 was considered the LLD. The LLD was equivalent or lower in the triplex real-time PCR (7578 CFU/ml for *lytA*, 805 CFU/ml for *sodC* and 1645 CFU/ml for *hpd*) compared to the singleplex real-time PCR (11,495 CFU/ml for *lytA,* 1354 CFU/ml for *sodC* and 1271 CFU/ml for *hpd*). A previous study comparing a triplex assay to singleplex assays for the detection of Sp, Nm and Hi by real-time PCR using extracted DNA showed that the LLD for the triplex were similar to that of the singleplex, ranging from 250 to 52,500 CFU equivalents per milliliter (Wang et al., 2012). These values show that there is no loss of sensitivity in the triplex direct real-time PCR compared to the singleplex direct real-time PCR.

A side-by-side comparison between the singleplex and triplex direct real-time PCR was conducted using CSF specimens that were collected from Brazil (N = 46), Burkina Faso (N = 109) and Niger (N = 45). All these specimens have been previously tested using singleplex real-time PCR and/or bacterial culture. They include 50 *lytA*-positive, 50 *sodC*-positive, 50 *hpd*-positive and 50 CSF that were negative for all three targets. The agreement between the two assays was assessed using Bland–Altman plot (Fig. 1), where the Ct difference (ΔCt) between the triplex and the singleplex ($Ct_{triplex} - Ct_{singleplex}$) for each target is represented as a function of the average Ct values (Bland and Altman, 1986). The upper and lower limits of agreement for each target were calculated as the Mean ΔCt ± 2 standard deviations. If the two methods agree, we expect 95% of the ΔCt to fall within the limits of agreement. Only two ΔCt values (4%) were outside the limits of agreement for *lytA* (Fig. 1A) and for *sodC* (Fig. 1B), and all ΔCt values for *hpd* were within the limits of agreement (Fig. 1C). Moreover, pairwise comparison of the singleplex and the triplex reaction Ct values was also completed using kappa statistics (Cohen, 1960). The calculated kappa value for each of the gene targets was equal to one, confirming a very good strength of agreement between the singleplex and the triplex direct PCR. All 50 negative specimens were found to be negative by both real-time PCR methods, and none of the positive specimens was discordant between the two methods. These results confirmed that the direct multiplex PCR detects the meningitis pathogens with the same sensitivity and specificity as the singleplex.

Bacterial meningitis is a vaccine preventable disease with a high morbidity and mortality worldwide (Weisfelt et al., 2006a,b). Bacterial meningitis epidemics are recurrent in some parts of the world with high socioeconomic consequences (Mohammed et al., 2017). The PCR methods used currently for the detection of the bacterial pathogens from CSF either rely on DNA extraction or use multiple singleplex reactions. The throughput of the methods that rely on DNA extraction can be very limiting due to this laborious and time-consuming step, particularly during large meningitis outbreaks, where laboratories can receive hundreds of specimens that require testing in a limited time frame. The newly validated triplex direct real-time PCR presented here addresses these challenges by allowing the simultaneous detection of Sp, Nm, and Hi directly from CSF in a single reaction without performing DNA extraction. Overall, the triplex direct real-time PCR assay detected the major meningitis pathogens in clinical specimens with similar sensitivity and specificity compared to the singleplex assays. The triplex direct real-time PCR assay saves valuable specimen volume, reagents, and processing time. The triplex direct real-time PCR was successfully used recently to respond quickly to a meningitis outbreak, allowing for timely identification of bacterial pathogens in several hundreds of specimens and subsequent rapid intervention (Aku et al., 2017).

Conflict of interest

There is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2018.10.008.

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