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Clinical Validation of Multiplex Real-Time PCR Assays for Detection of Bacterial Meningitis Pathogens

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***Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* are important causes of meningitis and other infections, and rapid, sensitive, and specific laboratory assays are critical for effective public health interventions. Singleplex real-time PCR assays have been developed to detect *N. meningitidis ctrA*, *H. influenzae hpd*, and *S. pneumoniae lytA* and serogroup-specific genes in the *cap* locus for *N. meningitidis* serogroups A, B, C, W135, X, and Y. However, the assay sensitivity for serogroups B, W135, and Y is low. We aimed to improve assay sensitivity and develop multiplex assays to reduce time and cost. New singleplex real-time PCR assays for serogroup B *synD*, W135 *synG*, and Y *synF* showed 100% specificity for detecting *N. meningitidis* species, with high sensitivity (serogroup B *synD*, 99% [75/76]; W135 *synG*, 97% [38/39]; and Y *synF*, 100% [66/66]). The lower limits of detection (LLD) were 9, 43, and 10 copies/reaction for serogroup B *synD*, W135 *synG*, and Y *synF* assays, respectively, a significant improvement compared to results for the previous singleplex assays. We developed three multiplex real-time PCR assays for detection of (i) *N. meningitidis ctrA*, *H. influenzae hpd*, and *S. pneumoniae lytA* (NHS assay); (ii) *N. meningitidis* serogroups A, W135, and X (AWX assay); and (iii) *N. meningitidis* serogroups B, C, and Y (BCY assay). Each multiplex assay was 100% specific for detecting its target organisms or serogroups, and the LLD was similar to that for the singleplex assay. Pairwise comparison of real-time PCR between multiplex and singleplex assays showed that cycle threshold values of the multiplex assay were similar to those for the singleplex assay. There were no substantial differences in sensitivity and specificity between these multiplex and singleplex real-time PCR assays.**

Approaches for detection of bacterial meningitis pathogens are continuously being improved. Detection of the specific serogroup or serotype causing disease can lead to better understanding of disease epidemiology, which is essential for planning appropriate vaccination programs and monitoring their impact. *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* are human commensal bacteria that also cause a spectrum of invasive diseases that include not only meningitis but also pneumonia and sepsis (8, 14, 24, 25, 29). Each of the three organisms is categorized by the structure of the polysaccharide capsule into different serogroups or serotypes. Six *N. meningitidis* serogroups (A, B, C, W135, X, and Y), six *H. influenzae* serotypes (a, b, c, d, e, and f), and 23 *S. pneumoniae* serotypes are commonly associated with invasive disease (14, 15). *N. meningitidis* and *S. pneumoniae* are considered the leading causes of bacterial meningitis worldwide after the implementation of the *H. influenzae* type b (Hib) conjugate vaccine (16, 18–20, 26). The serotype or serogroup distributions of the three pathogens vary by geographic region (8, 16, 27).

Singleplex real-time PCR assays have been developed to rapidly detect *N. meningitidis ctrA* (the capsule transport gene), *H. influenzae hpd* (the protein D gene), and *S. pneumoniae lytA* (the autolysin gene) (4, 6, 22, 28). Singleplex real-time PCR assays are available to determine *N. meningitidis* serogroups by detecting serogroup-specific genes in the *cap* locus (serogroup A *sacB*, W135 *synG*, X *xcbB*, B *synD*, C *synE*, and Y *synF*) (22). However, the assay sensitivity for serogroups B, W135, and Y is low. We aimed to improve the sensitivity of the singleplex real-time PCR assays for serogroup B *synD*, W135 *synG*, and Y *synF* by redesigning the primers and probes. Bacterial detection using singleplex real-time PCR requires considerable time and costly reagents, especially when handling large quantities of specimens. To reduce the cost and time for detecting the causative bacteria in settings such as meningitis outbreaks and epidemics, we developed and validated three multiplex real-time PCR assays for the detection of (i) *N. meningitidis ctrA*, *H. influenzae hpd*, and *S. pneumoniae lytA* (NHS assay), (ii) *N. meningitidis* serogroups A, W135, and X (AWX assay), and (iii) *N. meningitidis* serogroups B, C, and Y (BCY assay), which are the major causes of meningococcal diseases in the United States.

MATERIALS AND METHODS

Bacterial strains. The clinical isolates of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* used in this study were collected from 1997 to 2008 as part of the Active Bacterial Core surveillance (ABCs) of the Centers for Disease Control and Prevention's Emerging Infections Program (http://www.cdc.gov/abcs/index.htm). These isolates were collected from culture-confirmed cases of meningitis, pneumonia, or bacteremia. The *N. meningitidis* serogroup or *H. influenzae* serotype of each isolate was deter-

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mined by slide agglutination as part of the routine testing. The *S. pneumoniae* serotypes were determined by PCR (10). Reference strains were purchased from the American Type Culture Collection (ATCC).

Growth conditions. All *H. influenzae* isolates were grown on chocolate II agar supplemented with hemoglobin and IsoVitalex (BD, Sparks, MD) and incubated at 37°C for 18 to 24 h with 5% CO_2. All *N. meningitidis* and *S. pneumoniae* isolates were grown on TSA II agar plates supplemented with 5% sheep blood (BD, Sparks, MD) and incubated at 37°C for 18 to 24 h with 5% CO_2.

DNA extraction. For isolates and clinical specimens from Brazil, DNA was extracted as described previously (4). For clinical specimens and transport medium samples that did not yield viable cultures (nonviable transport medium [NVTM] samples) from South Africa and specimens from nasal washes or throat swabs, DNA was extracted using a MagNA pure compact nucleic acid isolation kit I (Roche Diagnostics, Mannheim, Germany) with a MagNA pure compact instrument (Roche Applied Science, Mannheim, Germany). A known specimen (positive control) and a water sample (negative control) were also extracted each time the DNA extraction procedure was performed on unknown specimens, which ensures that the negative real-time PCR results were not due to DNA extraction failure and that the positive PCR results were not due to cross-contamination introduced during the DNA extraction process. To prepare crude cell lysates, bacterial cultures grown overnight on appropriate agar plates were collected and suspended in 1 ml of 10 mM Tris buffer (pH 8.0). The cell suspensions were boiled for 10 min and stored at −20°C.

Real-time PCR and lower limit of detection (LLD). Real-time PCR was performed as described previously (22) using Invitrogen Platinum quantitative PCR Supermix-UDG master mix and run on either a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA) or an Applied Biosystems 7500 instrument (Applied Biosystems, Santa Clara, CA). PCR assembly and cycling conditions are described in Table S1 in the supplemental material.

To determine the LLD of each real-time PCR assay run on the Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA), genomic DNA was extracted from clinical isolates, and the DNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and adjusted to 20 ng/μl; DNA was then serially diluted in 10-fold increments. Each DNA concentration was converted to a genome equivalent per microliter using a standard of 2.2 Mb per *N. meningitidis* genome, 1.83 Mb per *H. influenzae* genome, or 2.1 Mb per *S. pneumoniae* genome. The LLD for a real-time PCR assay was defined as the DNA concentration that yielded a cycle threshold (C_T) value of 35.

Development of new singleplex real-time PCR assays for *N. meningitis* serogroup B *synD*, W135 *synG*, and Y *synF*. Primer Express 3.0 (Applied Biosystems) was used to design appropriate primers and probes for the singleplex real-time PCR assays based on published sequences of *synD* in *N. meningitidis* B, *synG* in *N. meningitidis* W135, and *synF* in *N. meningitidis* Y (22). All primers and probes were optimized by testing concentrations in the range of 100 nM to 900 nM and 100 nM to 400 nM, respectively, except for the *lytA* real-time PCR assay (see Table S2 in the supplemental material). The *lytA* assay was performed as described previously (4), with modifications (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm) (6). Fifty-eight isolates of non-*N. meningitidis* species collected through the ABCs or from the ATCC were tested to determine whether the new real-time PCR assays were specific for detecting the species *N. meningitidis* (Table 1; also see Table S3 in the supplemental material). The capability of the three new singleplex real-time PCR assays for detecting the specific *N. meningitidis* serogroup was evaluated by testing a convenience sample of 227 ABCs clinical isolates, including 22 serogroup A, 76 serogroup B, 24 serogroup C, 39 serogroup W135, and 66 serogroup Y isolates. The C_T value of each new assay was compared with that of the corresponding previously developed assay by testing 61 ABCs *N. meningitidis* isolates, including serogroups B, W135, and Y. All real-time PCRs were run on a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA).

TABLE 1 Detection of nontarget bacterial species by the real-time PCR assays in multiplex and singleplex[a]

Assay	No. negative/total no. (%)	95% CI[b]
N. meningitidis ctrA	58/58 (100)	93–100
H. influenzae hpd	60/60 (100)	94–100
S. pneumoniae lytA	66/66 (100)	95–100
N. meningitidis serogroup	58/58 (100)	93–100

[a] The negative rates for each assay were identical in multiplex and singleplex. The isolates tested are listed in Table S3 in the supplemental material.
[b] 95% CI, 95% confidence interval.

Validation of the three multiplex real-time PCR assays using ABCs isolates. Sixty-seven isolates representing different bacterial species collected through the ABCs or from the ATCC were tested to confirm that the multiplex and singleplex real-time PCR assays were specific for detecting the target bacterial species (see Table S3 in the supplemental material). The three multiplex real-time PCR assays (NHS, AWX, and BCY assays) were then evaluated using a convenience sample of 109 ABCs clinical isolates. The NHS assay was tested using 80 ABCs isolates, including 31 *H. influenzae*, 23 *N. meningitidis*, and 26 *S. pneumoniae* isolates. The serogroup-specific AWX and BCY assays were tested using 52 ABCs isolates, including 8 serogroup B, 9 serogroup C, 10 serogroup Y, 9 serogroup A, 7 serogroup X, and 9 serogroup W135 isolates. The 23 *N. meningitidis* isolates used for the NHS assay validation were included in the 52 isolates used for validating the *N. meningitidis* serogroup-specific AWX and BCY assays. To test a wide range of template DNA concentrations, crude DNA prepared from these isolates was diluted (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}); each DNA dilution was tested using the real-time PCR assays in both multiplex and singleplex platforms. All real-time PCRs were run on a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA). The C_T values of each real-time PCR assay between multiplex and singleplex platforms were compared by pairwise comparison analysis. Dilutions that did not give any C_T value were excluded from the analysis. In addition, pairwise comparison between duplicate singleplex assays for *N. meningitidis ctrA*, *H. influenzae hpd*, *S. pneumoniae lytA*, and serogroup B *synD* was also conducted to evaluate intra-assay variation (see Tables S4 and S5 in the supplemental material).

Clinical validation of multiplex real-time PCR NHS assay using specimens from routine surveillance for invasive disease caused by *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* in South Africa. A total of 121 specimens from South Africa (67 cerebrospinal fluid [CSF] specimens, 26 blood specimens, and 28 NVTM samples) were used to validate the NHS assay in multiplex and singleplex platforms on an Applied Biosystems 7500 instrument (Applied Biosystems, Santa Clara, CA). The clinical specimens were collected from patients of all ages from January 2008 through April 2009 as part of routine national laboratory-based surveillance for invasive disease due to *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*. Clinical specimens and NVTM samples, with clinical and demographic data, were sent to the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa, from approximately 120 laboratories throughout South Africa. NVTM samples refer to transport medium samples that yielded no culture in the reference laboratory at the NICD. Brain heart infusion (BHI) broth was added to the nonviable transport medium samples, which were incubated overnight at 37°C with 5% CO_2 for DNA extraction. A composite reference standard (positive defined as culture or latex agglutination positive; negative otherwise) was used to compare the sensitivities and specificities among the multiplex and singleplex real-time PCR assays (2).

Clinical validation of real-time PCR *H. influenzae hpd* assay in multiplex and singleplex using nasal washes and/or throat swabs from patients with respiratory infections at Lackland Air Force Base. A total of 487 nasal washes and/or throat swabs were collected from 255 patients with upper respiratory infections as part of infectious disease surveillance at Lackland Air Force Base in San Antonio, TX. Nasal washes were collected in ap-

TABLE 2 Detection of *N. meningitidis* serogroups by the new singleplex real-time PCR assays[a]

Assay	Result for target *N. meningitidis* serogroup		No. negative/no. tested for other *N. meningitidis* serogroups
	No. positive/total no. (%)	95% CI	
Serogroup B *synD*	75/76 (99)	93–100	0/151
Serogroup W135 *synG*	38/39 (97)	87–100	0/188
Serogroup Y *synF*	66/66 (100)	95–100	0/161

[a] A total of 227 *N. meningitidis* isolates were tested, including 22 serogroup A, 76 serogroup B, 24 serogroup C, 39 serogroup W135, and 66 serogroup Y isolates.

TABLE 3 Lower limits of detection of the real-time PCR assays

Assay	Organism	Genome DNA equivalents/PCR		
		Multiplex[a]	New singleplex	Previous singleplex
N. meningitidis ctrA	*N. meningitidis* B	18	23	NA[b]
	N. meningitidis Y	14	12	NA
H. influenzae hpd	Hib	42	46	NA
	HiNT[c]	23	23	NA
S. pneumoniae lytA	*S. pneumoniae* type 4	1	1	NA
	S. pneumoniae type 6B	1	1	NA
Serogroup B *synD*	*N. meningitidis* B	9	9	66,171
Serogroup C *synE*	*N. meningitidis* C	29	17	NA
Serogroup Y *synF*	*N. meningitidis* Y	11	10	8,955
Serogroup A *sacB*	*N. meningitidis* A	210	221	NA
Serogroup W135 *synG*	*N. meningitidis* W135	48	43	1,088,161
Serogroup X *xcbB*	*N. meningitidis* X	11	20	NA

[a] The multiplex assays were the NHS assay, detecting *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*; the BCY assay, detecting *N. meningitidis* serogroups B, C, and Y; and the AWX assay, detecting *N. meningitidis* serogroups A, W135, and X.
[b] NA, not applicable.
[c] HiNT, nontypeable *H. influenzae*.

proximately 2 ml saline; throat swabs were stored in 5 ml BD viral transport medium. Genomic DNA was extracted from 400 μl of nasal wash or throat swab specimens and tested with various PCR assays. Each specimen was screened for a variety of viral and bacterial pathogens, including *H. influenzae*, at Lackland Air Force Base (21). A high rate of *H. influenzae* in patients with viral infections was identified by use of a conventional PCR assay targeting the *fucK* gene (23). These specimens were used to validate the *H. influenzae hpd* real-time PCR assay in multiplex (NHS assay) and singleplex platforms using an Applied Biosystems 7500 instrument (Applied Biosystems, Santa Clara, CA). The *fucK* PCR assay was used as the reference standard for comparison of the sensitivities and specificities of the *H. influenzae hpd* assay between multiplex and singleplex platforms.

Clinical validation of real-time PCR assays for *N. meningitidis ctrA* and serogroup C *synE* in multiplex and singleplex using clinical specimens from meningitis patients in Brazil. CSF specimens from patients of all ages were collected from routine bacterial meningitis surveillance from 2007 to 2008 in Sao Paul, Brazil. A convenience sample of 100 CSF specimens was sent to the CDC in Atlanta, GA, as part of a quality control program agreed upon between the CDC and the Adolfo Lutz Institute in Sao Paul, Brazil, and was used to validate the *N. meningitidis ctrA* and serogroup C *synE* real-time PCR in multiplex (NHS and BCY) and singleplex assays using a Stratagene Mx3005P instrument (Agilent Technology, Santa Clara, CA). Since culture or latex agglutination results were not available for these CSF specimens, counterimmunoelectrophoresis (CIE) (13) conducted at the Adolfo Lutz Institute was used as the reference standard to compare the sensitivities and specificities of the *N. meningitidis ctrA* and serogroup C *synE* assays between multiplex and singleplex assays.

Statistical analysis. For each pairwise comparison (between multiplex and singleplex or between singleplex duplicates), the Bland-Altman plot was used to depict the magnitude of disagreement between the two assays ($\Delta C_{T\text{multiplex-singleplex}}$ [$\Delta C_{T\text{m-s}}$] and $\Delta C_{T\text{singleplex1-singleplex2}}$ [$\Delta C_{T\text{s1-s2}}$]) as a function of the average C_T value of the two assays [($C_{T\text{multiplex or singleplex}} + C_{T\text{singleplex}}$)/2]. The reference lines on the plot indicated the ideal zero difference and the observed mean C_T difference (mean $\Delta C_{T\text{m-s}}$ or mean $\Delta C_{T\text{s1-s2}}$), as well as the upper and lower limits of agreement defined by the mean C_T difference ± 1.96 standard deviations (SD). If the C_T differences ($\Delta C_{T\text{m-s}}$ or $\Delta C_{T\text{s1-s2}}$) are normally distributed, then 95% of the C_T differences (ΔC_T) would fall between the lower and upper limits of the agreement (1–3). Summary statistics were calculated for each assay, including sample size (n), mean C_T, SD, minimum C_T, median C_T, and maximum C_T.

The sensitivity and specificity of multiplex and singleplex platforms for 4 real-time PCR assays (*N. meningitidis ctrA*, *H. influenzae hpd*, *S. pneumoniae lytA*, and serogroup C *synE*) were assessed using the specimens collected from Lackland Air Force Base, Brazil, and South Africa. Other real-time PCR assays were not assessed due to the insufficient sample size (see Table S6 in the supplemental material). For all PCR assays, a specimen was considered positive if its C_T value was ≤35 and negative if its C_T value was >40. If a C_T value was >35 and ≤40, the specimen was diluted 10-fold and retested to determine if PCR inhibitors were present. The specimen was considered positive if the C_T value of the diluted specimen was ≤35 and negative if the C_T value was >35. The specimens that were negative by real-time PCR were tested for the presence of an RNase P-encoding gene to exclude PCR inhibition and DNA extraction failure. The sensitivity of a real-time PCR assay was defined as the proportion of real-time PCR-positive cases (multiplex or singleplex) out of positive cases determined by a reference standard [number of true positives/(number of true positives + number of false negatives)]. The specificity of a real-time PCR assay was defined as the proportion of real-time PCR-negative cases out of the negative cases determined by a reference standard [number of true negatives/(number of true negatives + number of false positives)]. Analyses were performed using SAS 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Improved singleplex real-time PCR assays for detection of *N. meningitidis* serogroups B, W135, and Y. To improve the sensitivity of *N. meningitidis* serogroup determination, we redesigned or modified the primers and probes for real-time PCR detection of *N. meningitidis* serogroups B, W135, and Y, based on published DNA sequences of the *synD*, *synG*, and *synF* genes, respectively (see Table S2 in the supplemental material) (22). The three new singleplex real-time PCR assays did not amplify DNA from any non-*N. meningitidis* species (Table 1; also see Table S3 in the supplemental material). Each assay was 100% specific for detection of its target serogroup (Table 2). The positive rates for detection of the target serogroup were 99%, 97%, and 100% for assays detecting serogroup B *synD*, W135 *synG*, and Y *synF*, respectively. The lower limit of detection (LLD) of each assay was significantly improved compared to that of the corresponding previously developed assay (Table 3). C_T values of the new assays were also compared to those of the previously developed assays by testing an additional 21 serogroup B, 11 serogroup W135, and 29 serogroup Y isolates. Each new assay showed lower C_T values than the previ-

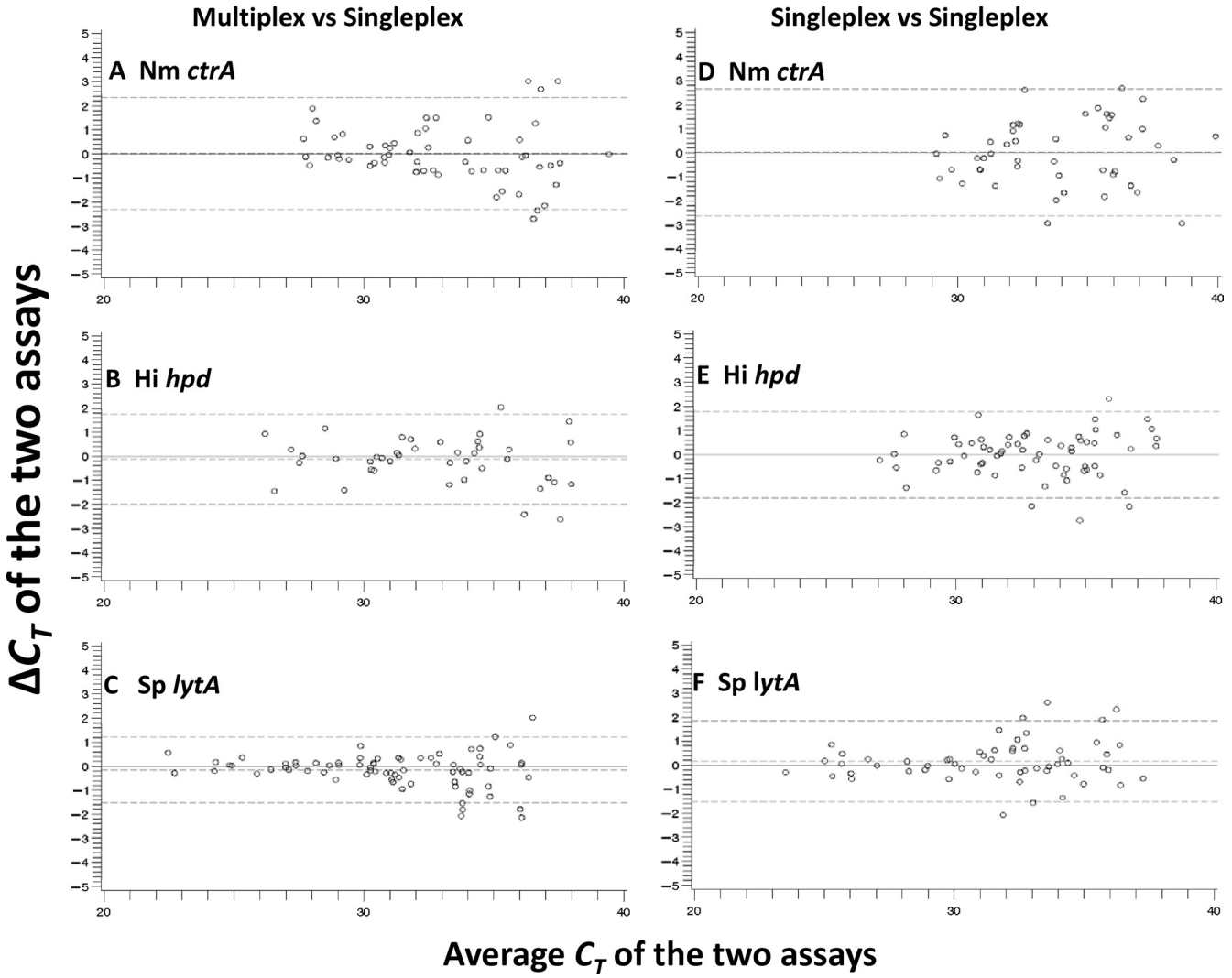

FIG 1 Pairwise comparison analyses for species-specific PCR assays for *N. meningitidis* (Nm) *ctrA*, *H. influenzae* (Hi) *hpd*, and *S. pneumoniae* (Sp) *lytA* carried out using ABCs isolates. Each assay was compared between multiplex and singleplex (A, B, and C) and between singleplex duplicates (D, E, and F). Shown is the Bland-Altman plot that depicts the cycle threshold difference (ΔC_T) between the two assays ($\Delta C_{T\text{multiplex-singleplex}}$ [$\Delta C_{T\text{m-s}}$] or $\Delta C_{T\text{singleplex1-singleplex2}}$ [$\Delta C_{T\text{s1-s2}}$]) as a function of the average C_T value of the two assays [($C_{T\text{multiplex or singleplex}} + C_{T\text{singleplex}}$)/2]. The reference lines on the plot indicate the ideal zero difference (solid line) and the observed mean C_T difference (mean $\Delta C_{T\text{m-s}}$ or mean $\Delta C_{T\text{s1-s2}}$) (middle dashed line), as well as the upper and lower limits of agreement defined by the mean $\Delta C_T \pm 1.96$ standard deviations (top and bottom dashed lines) (1–3).

ously developed assay, with a difference in C_T value of 4 to 28 cycles for all isolates tested (data not shown).

Development of multiplex real-time PCR assays (NHS, AWX, and BCY). Fluorescence-labeled probes were tested for each multiplex real-time PCR assay. Table S2 in the supplemental material shows the optimal combinations of probe-reporter dye for each multiplex assay. Each of the nine assays was 100% specific for detection of its target organism in both multiplex and singleplex (Table 1). The LLD of each assay in multiplex was similar to that in singleplex (Table 3). Seven of the assays were able to detect low numbers of DNA copies per PCR (less than 50 genome equivalents). The serogroup A *sacB* assay detected higher genome equivalents than the other assays tested in this study.

Pairwise comparison of real-time PCR assays between multiplex and singleplex platforms and between singleplex assay duplicates. Pairwise comparison of the NHS, AWX, and BCY assays between multiplex and singleplex was first conducted using clinical isolates. $\Delta C_{T\text{m-s}}$ of these assays was within a range of −3.7 to 4.9 cycles (Fig. 1 and 2; also see Table S4 in the supplemental material). Pairwise comparison between duplicate singleplex assays for *N. meningitidis ctrA*, *H. influenzae hpd*, and *S. pneumoniae lytA* (Fig. 1) and serogroup B *synD* (data not shown) was also conducted to evaluate intra-assay variation. $\Delta C_{T\text{s1-s2}}$ was within a similar range, from −2.9 to 3.4 (see Table S5 in the supplemental material). For each assay, both the C_T differences and the standard deviations (SD) of the C_T differences were similar between the multiplex-singleplex and singleplex-singleplex comparisons, indicating that the measurement errors were similar between the multiplex and singleplex platforms of these assays. In general, for each assay, the level of agreement between multiplex and singleplex was similar to that between singleplex duplicates, with most C_T differences in each comparison falling within the limits of

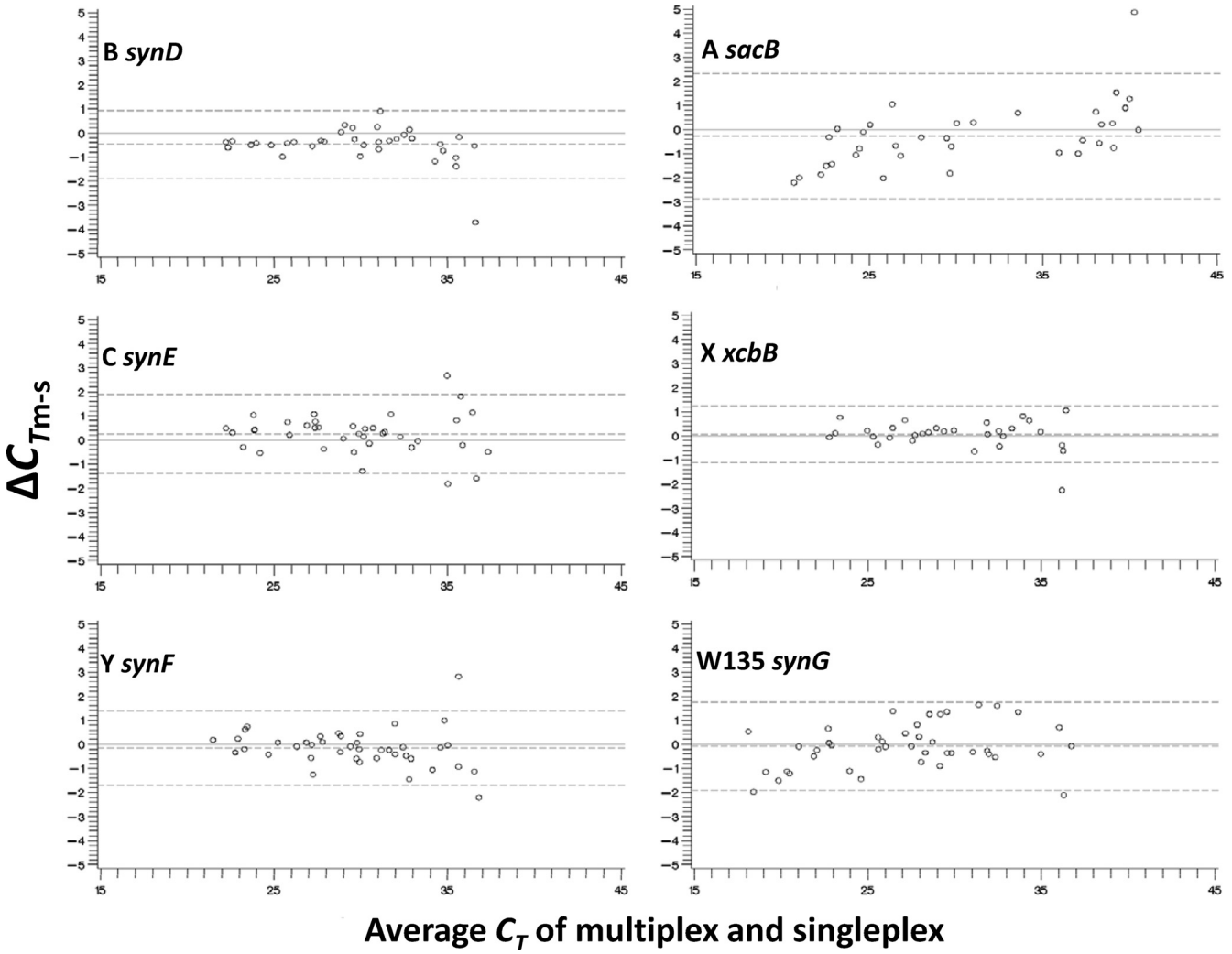

FIG 2 Pairwise comparison analyses for *N. meningitidis* serogroup-specific PCR assays carried out using ABCs isolates. Each of the six PCR assays was compared between multiplex and singleplex platforms. The Bland-Altman plot for each assay is shown.

agreement (Fig. 1). The ΔC_T of PCR for most DNA specimens tested was within the lower and upper limits of agreements of −2.9 and 2.3 cycles, respectively, between multiplex and singleplex and −2.6 and 2.7, respectively, between singleplex duplicates (see Tables S4 and S5). A few outlier results with greater $\Delta C_{Tm\text{-}s}$ or $\Delta C_{Ts1\text{-}s2}$ values were observed when the DNA concentration was low (C_T value of >35). The mean $\Delta C_{Tm\text{-}s}$ value of each assay was very close to zero; there are not substantial differences in real-time PCR between the multiplex and singleplex platforms.

$\Delta C_{Tm\text{-}s}$ was also determined by testing clinical specimens from South Africa, Brazil, and Lackland Air Force Base (see Fig. S1 and Table S6 in the supplemental material). Extracted DNA from these specimens was tested without dilution. Only specimens that were positive by the reference standard test were included in the pairwise comparison (see Fig. S1 and Table S6). The greatest $\Delta C_{Tm\text{-}s}$ values were observed for the *H. influenzae hpd* assay using nasal washes and/or throat swabs collected at Lackland Air Force Base, but only 13 values (4.9%) were outside the limits of agreement (see Fig. S1). Although few specimens from South Africa were tested with the *H. influenzae hpd* and *S. pneumoniae lytA* assays, the level of agreement between multiplex and singleplex was the same as that observed when the U.S. clinical isolates were tested. However, a significantly lower average C_T value (mean difference of −1.7, paired *t* test, $P < 0.01$) was observed in the multiplex assay than in the singleplex assay for the specimens from South Africa when tested with *N. meningitidis ctrA* (see Fig. S1). Similarly, when the clinical specimens from Brazil were tested with the serogroup C *synE* assay, a lower average C_T value (mean difference of −1.3, paired *t* test, $P < 0.01$) was observed using the multiplex assay than using the singleplex (see Fig. S1).

Sensitivity and specificity of multiplex and singleplex real-time PCR assays for *N. meningitidis ctrA*, *H. influenzae hpd*, *S. pneumoniae lytA*, and serogroup C *synE*. Different reference standards (*fucK* PCR, CIE, and culture/latex composite reference standards) were used to assess the sensitivity and the specificity of real-time PCR assays for *N. meningitidis ctrA*, *H. influenzae hpd*, *S. pneumoniae lytA*, and serogroup C *synE* in multiplex and singleplex platforms. As shown in Table 4, there was no significant difference in sensitivity and specificity between the multiplex and singleplex real-time PCR assays.

TABLE 4 Sensitivities and specificities of the multiplex and singleplex real-time PCR assays for detection of meningitis pathogens from clinical specimens

Specimen group (reference standard)	Assay (type)[a]	No. positive/no. tested	Sensitivity (95% CI)	No. negative/no. tested	Specificity (95% CI)
Lackland (*fucK* PCR)	*H. influenzae hpd* (M)	206/244	84.4 (79.3–88.7)	217/243	89.3 (84.7–92.9)
	H. influenzae hpd (S)	206/244	84.4 (79.3–88.7)	218/243	89.7 (85.2–93.2)
Brazil (CIE[b])	*N. meningitidis ctrA* (M)	72/75	96.0 (88.8–99.2)	8/24	33.3 (15.6–55.3)
	N. meningitidis ctrA (S)	72/75	96.0 (88.8–99.2)	9/24	37.5 (18.8–59.4)
	Serogroup C *synE* (M)	71/75	94.7 (86.9–98.5)	17/24	70.8 (48.9–87.4)
	Serogroup C *synE* (S)	72/75	96.0 (88.8–99.2)	17/24	70.8 (48.9–87.4)
South Africa (composite[c])	*N. meningitidis ctrA* (M)	56/76	73.7 (62.3–83.1)	43/44	97.7 (88.0–99.9)
	N. meningitidis ctrA (S)	58/76	76.3 (65.2–85.3)	37/44	84.1 (69.9–93.4)
	H. influenzae hpd (M)	16/21	76.2 (52.8–91.8)	98/99	99.0 (94.5–100.0)
	H. influenzae hpd (S)	18/21	85.7 (63.7–97.0)	91/99	91.9 (84.7–96.4)
	S. pneumoniae lytA (M)	16/17	94.1 (71.3–99.8)	102/103	99.0 (94.7–100.0)
	S. pneumoniae lytA (S)	16/17	94.1 (71.3–99.8)	96/103	93.2 (86.5–97.2)

[a] M, multiplex; S, singleplex.
[b] One specimen was not tested by CIE and was therefore excluded from this analysis.
[c] Combined laboratory tests (culture and/or latex agglutination) were used as the composite reference standard, where a positive result was defined as culture or latex agglutination positive (negative otherwise). One specimen was not tested by culture or latex agglutination and was therefore excluded from this analysis.

DISCUSSION

Real-time PCR is a powerful tool for detection of human pathogens because of the high sensitivity and throughput capability. Introduction of fluorescent probes improves the assay specificity and enables development of a multiplex platform for amplification and detection of multiple target genes. Multiplex real-time PCR allows simultaneous detection of multiple organisms in a single reaction, which conserves limited quantities of clinical specimens and significantly reduces costs, such as reagents and person time, which is particularly important in resource-limited settings. However, concerns that the sensitivity of a single assay may be compromised when it is run with other PCR assays in a single reaction remain. In this study, we found that the sensitivity of three multiplex real-time PCR assays was indistinguishable from that of the singleplex real-time PCR assays for detection of bacterial meningitis pathogens as evaluated using ABCs clinical isolates as well as clinical specimens collected from different geographic locations. The sensitivities and specificities of each assay varied among the different laboratories due to the use of different reference standards. Culture is still considered the "gold standard." Since none of the reference standards used in this study is considered the "gold standard," the sensitivity and specificity in this analysis may not be accurately assessed.

A multiplex real-time PCR assay using *N. meningitidis ctrA*, *H. influenzae bexA*, and *S. pneumoniae ply* as target genes has previously been developed (9). However, the *bexA* gene is present only in encapsulated *H. influenzae*, and the *bexA*-based real-time PCR assay is not able to detect nontypeable *H. influenzae*. Additionally, the *lytA* PCR assay has been shown to be more specific than the *ply* assay for detection of *S. pneumoniae* (4). The multiplex assay described in this study improves upon these previously developed assays by using the protein D gene as a target for detection of both typeable and nontypeable *H. influenzae* and the *lytA* gene as a target for detection of *S. pneumoniae* (4, 6, 28). Both multiplex real-time PCR assays use the *ctrA* gene as a target for detection of *N. meningitidis*. While the *ctrA*-based real-time PCR assay is a suitable tool for capturing most of the encapsulated *N. meningitidis* strains, it fails to detect strains that lack *ctrA* or that have significant variation in this gene (5, 7, 11). A recently developed real-time PCR assay targeting the *N. meningitidis sodC* gene can capture groupable and nongroupable *N. meningitidis* and is very useful for analyzing isolates or specimens collected from nasopharyngeal carriage surveys (12). The performance of the *sodC* assay in the multiplex platform remains to be investigated.

A CSF specimen from a meningitis patient typically has bacterial counts in excess of 10^3 to 10^5 CFU per milliliter (17). The LLDs of the multiplex real-time PCR assays described in this study range from 1 to 210 genome equivalents per real-time PCR (250 to 52,500 CFU equivalents per milliliter), which is within the range of bacterial counts in a meningitis CSF specimen. The real-time PCR assays described in this study should be able to detect the target pathogen from meningitis CSF specimens with typical bacterial load. This study used a C_T cutoff value of 35 for all real-time PCR assays. We recommend that specimens with high C_T values (>35 and ≤ 40) should be analyzed individually. We have observed that nonspecific amplification results in high C_T values and that specimens with high C_T values often have poor replicate reproducibility, which leads to higher rates of false positives (data not shown). High C_T values could also result from the presence of PCR inhibitors or target DNA degradation or low DNA quantity due to antibiotic treatment prior to CSF specimen collection or suboptimal specimen transport and storage conditions. Some of these possibilities can be evaluated by diluting specimens which have high C_T values or by performing antibiotic detection. In any case, interpretation of specimens with high C_T value should include consideration of clinical data and other laboratory results.

Several factors may influence the multiplex real-time PCR performance compared to the singleplex performance, including PCR reagents, DNA quality, and fluorescence reporter dyes. We observed lower sensitivity of some assays in multiplex than in singleplex when different commercial PCR reagents were used. In addition, the serogroup A *sacB* assay failed to detect *N. meningitidis* A when the probe was labeled with Cy5 in multiplex (data not shown) but performed well when the probe was labeled with Hex.

This underscores the importance of assay optimization when developing multiplex real-time PCR assays from the singleplex real-time PCR assays. The optimal conditions for singleplex real-time PCR may not be optimal for the multiplex PCR. The performance of the multiplex real-time PCR assay does not seem to be affected by different PCR instruments or methods used for DNA extraction.

The three multiplex real-time PCR assays described here have demonstrated sensitivities similar to that of the singleplex real-time PCR for detecting bacterial meningitis pathogens in clinical isolates, CSF specimens, blood, nasal washes, throat swabs, and inoculated transport medium specimens. Multiplex real-time PCR also conserves valuable resources, which makes it particularly useful in resource-limited settings. These advantages make the multiplex real-time PCR assays developed in this study a widely useful tool for disease surveillance and research into the causative pathogens of bacterial meningitis.

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