Development of a novel multiplex real-time PCR assay for detection of the four main causes of bacterial *meningitis*

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Introduction

Bacterial meningitis is a severe infection of the central nervous system (CNS) that poses significant health risks, including neurological deficits and epilepsy. Among the different types of meningitis, the bacterial form is particularly concerning, with *Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae* and *Streptococcus agalactiae* (GBS) being the most common and dangerous causative agents. The incidence of disease due to these pathogens varies greatly by region, with a higher burden observed in the African Meningitis Belt. Although, vaccination efforts have significantly reduced the incidence of bacterial meningitis, particularly through the introduction of conjugate vaccines, it is unlikely that a vaccine for every pathogenic strain will be available. Therefore, continuous vigilance is necessary to monitor circulating strains for adequate public health responses. This will require the development of improved diagnostic tools for detection of the main pathogens. In a recent study, we have investigated and identified alternative genes that are the most appropriate targets for the detection of *Streptococcus pneumoniae, SP2020* and *psaA; Neisseria meningitidis, sodC* and *porA; Haemophilus influenzae, dmsA* (*HAEM1183*) and *hpd; Streptococcus agalactiae, cfb* and *sip*.
This study aimed to develop a novel multiplex real-time PCR assay targeting *porA, dmsA, SP2020* and *cfb* genes to improve the diagnosis of *N. meningitidis, H. influenzae, S. pneumoniae* and *S. agalactiae*.

Material and methods

DNA samples

- 45 DNA samples used for multiplex real-time PCR assay of which 7 *S. agalactiae,* 7 *H. influenzae,* 8 *N. meningitidis* and 8 *S. pneumoniae*
  - ✓ 11 reference strains DNA extracts from the National Collection of Type Cultures (NCTC)
  - ✓ 34 DNA extracts from blood cultures, CSF, pleural fluid and patient tissue and carriage.

Genomic target and primers

Gene	Primer name	5' – 3' nucleotide sequence
cfb	cfb-F2	GAAACATTGATTGCCCAGC
	cfb-R2	AGGAAGATTTATCGCACCTG
	cfb-PB2	Cy3-CCATTTGATAGACGTTCGTGAAGAG-BHQ
porA	porA_fwd_1	GCCGGCGTTGATTATGATTT
	porA_rev_1	AGTTGCCGATGCCGGTATT
	porA_pb_1	JOE-CTTCCGCCATCGTGTC-BHQ
SP2020	SP_2020_F	TAAACAGTTTGCCTGTAGTCG
	SP_2020_R	CCCGGATATCTCTTTCTGGA
	SP_2020_P	Cy5-AACCTTTGTTCTCTCTCGTGGCAGCTCAA-BHQ
dmsA	HAEM1183_F	TATGGTACGGGAACACTCGG
	HAEM1183_R	ATTTCCCAATGCCCAACCAC
	HAEM1183_PB	FAM-GTGATTACAGCACCGCACAA-BHQ1

Monoplex real-time PCR assay and optimization

- Real-time PCR targeting *porA, cfb* and *SP2020* performed according to condition described in published paper
- PCR optimised by using a final concentration of 0.5 µM for primers and probe and PCR targeting new gene *dmsA* performed under the same condition
- The cycling parameters consisted of 2 minutes at 50°C, 10 minutes at 95°C, 45 cycles of 95°C for 15 s and 60°C for 1 min, and a holding stage at 4°C.
- Positive specimens defined as having Ct ≤ 35 for the assay,

Multiplex real-time PCR assay

- PCR targeting simultaneously *porA, cfb, SP2020* and *dmsA* using a final concentration of 0.25 µM for primers and probe
- Amplification performed under the same conditions as the monoplex assay.

Limit of detection (LLD)

- Reference strains DNA extracts *of N. meningitidis, H. influenzae, S. agalactiae* and *S. pneumoniae* were serial diluted 5 times, at a ratio of 1:10
- Multiplex PCR targeting *porA, cfb, SP2020* and *dmsA* performed on the diluted DNA
- Generation of a linear regression curve for each bacterium
- LLD corresponding to the lowest DNA concentration still detectable by the PCR assay.

Statistics

Sensitivity, specificity, negative predicted values (NPV) and positive predicted values (PPV) determined using formulas:

$$\text{Sensitivity (\%)} = \frac{\text{True positive} \times 100}{\text{True positive} + \text{false negative}} \qquad \text{Specificity (\%)} = \frac{\text{True negative} \times 100}{\text{True negative} + \text{false positive}}$$

$$\text{PPV (\%)} = \frac{\text{True positive} \times 100}{\text{True positive} + \text{false positive}} \qquad \text{NPV (\%)} = \frac{\text{True negative} \times 100}{\text{True negative} + \text{false negative}}$$

Results

Monoplex real-time PCR assay performance

- Sensitivity and NPV of the assay for detection of all pathogens was 100%.
- Specificity of the assay varied from 91.7% to 100% and PPV from 72.7% to 100% (Table 1).

Table 1: Monoplex Real-time PCR assays statistics

Target bacteria	Gene primer set	TP	FP	FN	TN	Se (%) (95% CI)	Sp (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
H. influenzae	*dmsA*	7	1	0	36	100 (59.0-100)	97.3 (85.8-99.9)	87.5 (47.3-99.7)	100 (90.3-100)
N. meningitis	*porA*	8	3	0	33	100 (63.1-100)	91.7 (77.5-98.2)	72.7 (39.0-94.0)	100 (89.4-100)
S. agalactiae	*Cfb*	7	0	0	37	100 (59.0-100)	100 (90.5-100)	100 (59.0-100)	100 (90.5-100)
S. Pneumoniae	*SP2020*	8	0	0	36	100 (63.1-100)	100 (90.3-100)	100 (63.1-100)	100 (90.3-100)

TP: True Positive; TN: True Negative; FP: False positive; FN: False Negative; Se: Sensibility; Sp: Specificity; CI: Confidence Interval, PPV: Positive predictive value, NPV: Negative predictive value

Multiplex real-time PCR assay performance

- Multiplex real-time PCR assays showed the same sensitivity, specificity, PPV and NPV as the monoplex assays for each of the four bacteria
  - ✓ Sensitivity and NPV of the assay for detection of all pathogens was 100%.
  - ✓ Specificity of the assay varied from 91.7% to 100% and PPV from 72.7% to 100% (Table 2).

Table 2: Sensitivity and specificity of the real time PCR multiplex assay

Target pathogen	TP/TP+FN	Se (%)	(95% CI)	TN/TN+FP	Sp (%)	(95% CI)
S. agalactiae (*cfb*)	7/7	100	59.0-100	37/37	100	90.5-100
H. influenzae (*dmsA*)	7/7	100	59.0-100	36/37	97.3	85.8-99.9
N. meningitidis (*porA*)	8/8	100	63.1-100	33/36	91.7	77.5-98.2
S. pneumoniae (*SP2020*)	8/8	100	63.1-100	36/36	100	90.3-100

TP: True Positive; TN: True Negative; FP: False positive; FN: False Negative; Se: Sensibility; Sp: Specificity; CI: Confidence Interval

Table 3: Positive and negative predictive values of the real time PCR multiplex assay

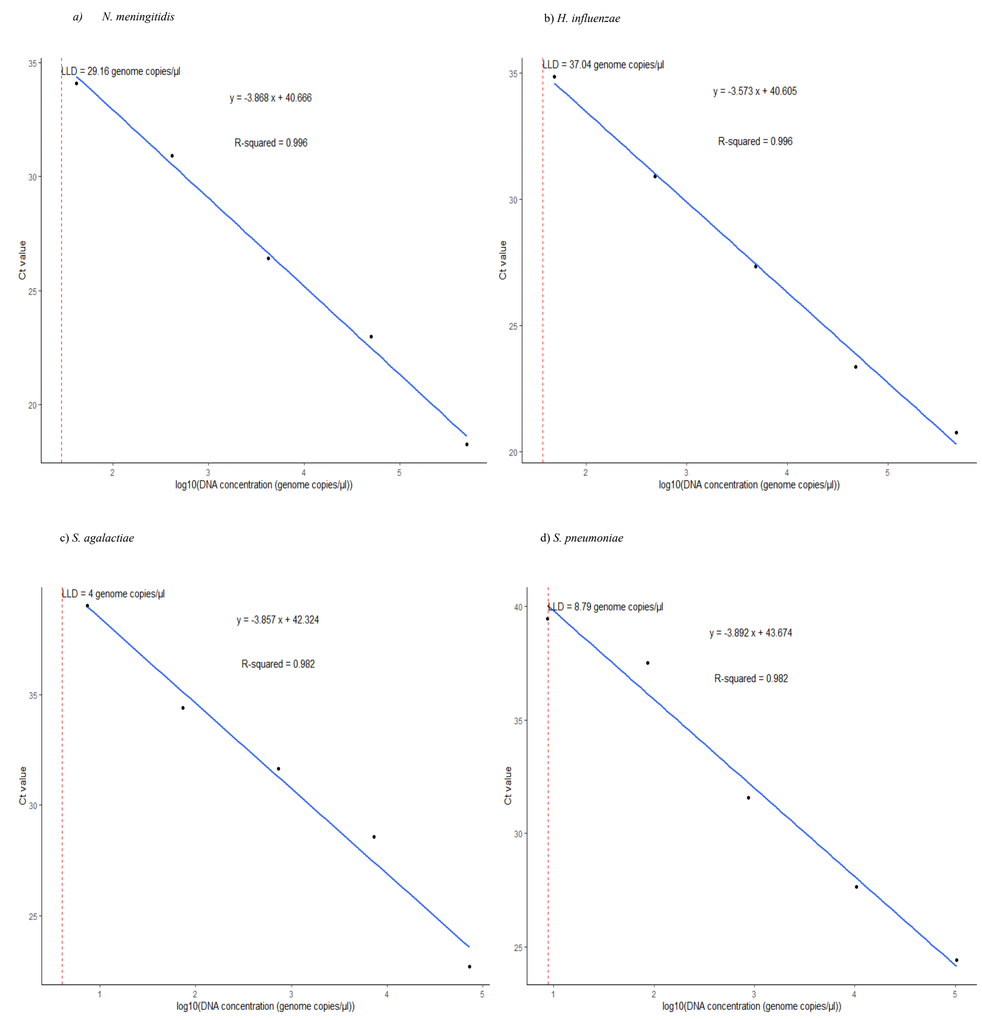
Target pathogen gene	TP/TP+FP	PPV (%)	(95% CI)	TN/TN+FN	NPV (%)	(95% CI)
S. agalactiae (*cfb*)	7/7	100	59.0-100	37/37	100	90.5-100
H. influenzae (*dmsA*)	7/8	87.5	47.3-99.7	36/36	100	90.3-100
N. meningitidis (*porA*)	8/11	72.7	39.0-94.0	33/33	100	89.4-100
S. pneumoniae (*SP2020*)	8/8	100	63.1-100	36/36	100	90.3-100

TP: True Positive; TN: True Negative; FP: False positive; FN: False Negative; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence Interval

Performance of the multiplex assay

- Standard curve characteristic
  - ✓ *N. meningitidis*: *porA* primers showing a slope (E) of -3.868, a correlation coefficient (R2 of 0.996), an efficiency (ε) of 81.36% and y-intercept (y) of 40.666
  - ✓ *H. influenzae*: *dmsA* primers showing a E = -3.573, R2 = 0.996, ε = 90.49% and y = 40.605
  - ✓ *S. agalactiae*: *cfb* primers showing a E = -3.857, R2 = 0.982, ε = 81.66% and y = 42.324 (Figure 1)
  - ✓ *S. pneumoniae*: *SP2020* primers showing a E= -3.892, R2 = 0.982, ε = 80.69% and y = 43.674
- Ct limit and LLD
  - ✓ Ct limit for *H. influenzae* and *N. meningitidis* determined to be 35 cycles corresponding to the LLD of 37 genome copies/µl and 29 genome copies/µl, respectively.
  - ✓ Ct limit for *S. agalactiae* and *S. pneumoniae* determined to be 40 cycles corresponding to the LLD of 4 genome copies/µl and 9 genome copies/µl, respectively.

Figure 1: Standard curve of the multiplex real-time PCR assay representing the ten-fold dilution series of DNA of a) *N. meningitis*, b) *H. influenzae*, c) *S. agalactiae* and d) *S. pneumoniae*.



Conclusion

The multiplex test developed as part of this study demonstrated high performance and efficacy. Although further validation is required, the test has demonstrated its potential for rapid and accurate screening for meningitis. The implementation of this test in low- and middle-income countries (LMICs) is essential to detect the agents responsible for meningitis and improve diagnostic capabilities. Extending the use of this test could provide accurate data on circulating bacterial strains, which could contribute to the development of effective prevention and control strategies.

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