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 to detect the pathogen’s main causes. In a recent study, we investigated and identified alternative genes that are the most appropriate targets for the detection of Streptococcus pneumoniae, SP2020 and psa, Neisseria meningitidis, sodC and porA, Haemophilus influenzae, dmNA (HpEM2183) and fha, Streptococcus agalactiae, cfb and sip.

This study aimed to develop a novel multiplex real-time PCR assay targeting porA, dmNA, SP2020 and cfb genes to improve the diagnosis of Neisseria 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 N. influenzae, 8 N. meningitidis and S. pneumoniae
• 21 reference strains DNA extracts from the National Collection of Type Cultures (NCTC)
• 54 DNA extracts from blood cultures, CSF, pleural fluid and patient tissue and carriage.

Monoplex real-time PCR assay and optimization

• Real-time PCR targeting porA, cfb and SP2020 performed according to protocol described in published paper
• PCR optimised by using a final concentration of 0.5 µM for primers and probe and PCR targeting new gene (s) performed under the same conditions.
• 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 dmNA using a final concentration of 0.25 µM for primers and probe
• Amplification performed under the same conditions as the monoplex assay.

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>
<thead>
<tr>
<th>Bacteria</th>
<th>Gene</th>
<th>TP</th>
<th>FN</th>
<th>TN</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis</td>
<td>dmNA</td>
<td>7</td>
<td>0</td>
<td>37</td>
<td>100 (95.1-100)</td>
<td>96.4 (92.0-99.7)</td>
<td>100 (93.0-100)</td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>porA</td>
<td>7</td>
<td>0</td>
<td>37</td>
<td>100 (95.1-100)</td>
<td>96.4 (92.0-99.7)</td>
<td>100 (93.0-100)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae</td>
<td>cfb</td>
<td>7</td>
<td>0</td>
<td>37</td>
<td>100 (95.1-100)</td>
<td>96.4 (92.0-99.7)</td>
<td>100 (93.0-100)</td>
<td></td>
</tr>
</tbody>
</table>

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: Specificity and sensitivity of the real-time PCR multiplex assay

<table>
<thead>
<tr>
<th>Target pathogen</th>
<th>TP/FP/FN</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>TN/TP+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis</td>
<td>7/7</td>
<td>100</td>
<td>59.0-100</td>
<td>37/37</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>7/7</td>
<td>100</td>
<td>59.0-100</td>
<td>37/37</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>8/8</td>
<td>100</td>
<td>63.1-100</td>
<td>32/32</td>
</tr>
<tr>
<td>S. agalactiae</td>
<td>8/8</td>
<td>100</td>
<td>63.1-100</td>
<td>32/32</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Target pathogen</th>
<th>TP/FP/FN</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>TN/TP+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis</td>
<td>7/8</td>
<td>100</td>
<td>59.0-100</td>
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<td>8/8</td>
<td>100</td>
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<td>32/32</td>
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</tbody>
</table>

Performance of the multiplex assay

• Standard curve characteristic:
  - N. meningitidis: porA primers showing a slope (E) of -3.688, a correlation coefficient (R²) of 0.996, an efficiency (a) of 91.36% and y-intercept (b) of 40.666
  - H. influenzae: dmNA primers showing a slope (E) = -3.579, R² = 0.996, x = 40.949 and y = 40.605
  - S. agalactiae: cfb primers showing a E = -3.857, R² = 0.982, x = 81.66% and y = 42.324 (Figure 1)
  - S. pneumoniae: SP2020 primers showing a E = -3.892, R² = 0.982, x = 80.69% and y = 43.674

• Ct limit and LLOD
  - Ct limit for H. influenzae and N. meningitidis determined to be 35 cycles corresponding to the LLOD 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 LLOD 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. meningitidis, 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.

Acknowledgements

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