**nspA** Gene as a specific genetic marker for detection of *Neisseria meningitidis* causing bacterial meningitis

Neha Bhatt¹, Nazneen Khan¹, Sandip K Dash¹, Shashi Khare² and Ashok Kumar¹*²

¹CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India
²National Centre for Disease Control, Sham Nath Marg, Delhi-110054, India

Received 07 March 2014; revised 12 May 2014

Bacterial meningitis caused by *Neisseria meningitidis* which causes human brain meninges damage, is generally diagnosed from patient cerebrospinal fluid through microscopy, immunological assays, biochemical test, PCR, microarray and biosensors. However, these methods are expensive, time-consuming or non-confirmatory due to certain limitations. A quick PCR based method was developed for detection of bacterial meningitis caused by *N. meningitidis* using specific primers based on amplification of virulence *nspA* (*Neisseria* surface protein A) gene partial sequence (202 bp). The *nspA* gene amplicon could be used as a genetic marker for minimum detection of 10 ng genomic DNA (G-DNA) of *N. meningitidis* with high sensitivity only in 80 min, which is least time reported for the confirmation of the disease. However, the lower detection limit was found as low as 1.0 ng G-DNA, but with less sensitivity. The cross-reactivity of the genetic marker was also studied with other possible pathogens. A comparison with the presently available detection methods and our method was also done using patient samples.

**Keywords**: Genetic marker, Meningitis, *Neisseria meningitidis*, *nspA* gene

Bacterial meningitis in human causes inflammation of the meninges in the brain and spinal cord, resulting in the damage of the brain or even death. The disease spreads very rapidly among people in contact, leading to endemic or epidemic outbreak. The conventional methods used for detection of human bacterial meningitis include biochemical tests¹, microscopy², latex agglutination test³, PCR⁴,⁵, RT-PCR⁶, microarray⁷,⁹ and biosensors¹⁰⁻¹³. However, these methods suffer from one or other limitations¹⁴. Therefore, a quick and specific method is required for diagnosis of the disease to prevent the outbreak and early treatment of the patients.

Bacterial meningitis has been detected using broad-range PCR of genes coding for 16s and 23s RNA of *N. meningitidis*, but it is not efficient due to its long proteolysis step of more than 12 h of incubation at 56°C¹⁵. Similarly, the multiplex PCR involves purification of RNA and synthesis of cDNA which is time-consuming¹⁶,¹⁷. Sero groups of *N. meningitidis* have also been detected by *crgA* and *siaD* gene sequence analysis, but is also time-consuming and complicated¹⁸,¹⁹. The purified genomic DNA (G-DNA) of *N. meningitidis* is also used as template for PCR²⁰,²¹. A multiple gene-based multiplex PCR is reported which is also complicated and expensive²². An eight-plex PCR method for simultaneous detection of *N. meningitidis*, *S. pneumoniae*, *E. coli*, *S. aureus*, *L. monocytogenes*, *S. agalactiae*, herpes simplex virus (types 1, 2) and varicella-zoster virus is also reported to detect PCR amplicon through array and microsphere coupling method, which require about a day for diagnosis of bacterial meningitis²³. Recently, *opc* gene and *omp85* gene as a genetic marker have been reported for detection of bacterial meningitis²⁴, but their amplicons are larger in size and take more time for detection.

The release of outer membrane vesicles that consist of lipooligosaccharides (endotoxin), outer membrane proteins, such as Neisseria surface proteins (*nspA*), phospholipids and capsular polysaccharides, is the major virulence factor of the *N. meningitidis*²⁵. The *nspA* is an ortholog gene in *N. meningitidis* and present in all serogroups, serotypes and subtypes. It is highly conserved among meningococcal strains and induces bactericidal antibodies, thus provides immune protection against *N. meningitidis* infection. *nspA* consists of an eight-stranded antiparallel β-barrel with β strands and is a homologue of the Opa proteins, which mediate adhesion to host cells²⁶. Here, we have
used nspA virulence gene amplicon of 202 bp as a genetic marker for quick diagnosis of *N. meningitidis* in patient samples of cerebrospinal fluid (CSF).

**Materials and Methods**

**Chemicals and instruments**

The PCR chemicals, Taq polymerase and agarose (Bangalore GeNei, Bangalore, India), EDTA (Qualigens, India) and Tris (hydroxymethyl)aminomethane (Sigma-Aldrich, USA) were used. DNA purification kit was purchased from Biochem Life Sciences, India. Forward and reverse primers were designed using Primer-3 program and bioinformatics tools and synthesized from GCC Biotech, India. The PCR purification GFX column was obtained from Biochem life Science, India. PCR was performed using thermal cycler MJ Mini (Bio-Rad), USA and quantification and purity of DNA was measured using NanoDrop 1000 spectro-photometer from USA.

*Neisseria meningitidis*, *Escherichia coli*, *Streptococcus pyogenes*, *S. pneumoniae*, *Salmonella typhi*, *Haemophilus influenzae* and *Mycobacterium tuberculosis* culture and patient cerebrospinal fluid (CSF) samples were obtained from Institute of Microbial Technology, Chandigarh, India and National Centre for Disease Control (NCDC), Delhi.

**PCR amplification**

Bacterial meningitis suspected patient CSF (0.5 ml) sample was taken in an eppendorf and centrifuged at 16,000 x g for 2 min. The supernatant was discarded and the pellet was suspended in 25 µl of PCR mix containing 1X PCR buffer (1.5 mM MgCl₂, 0.01 M Tris-HCl, 0.05 M KCl, 0.01% gelatin, pH 8.3), 1.0 mM dNTP mix (0.25 mM of each nucleotide), 1.5 mM MgCl₂, 0.4 µM of each forward (5´-CATCCACCGATTTCAAAC-3´) and reverse primer (5´-ACATTCCGGGTAACG GCA-3´) of nspA gene of *N. meningitidis*, 0.75 U Taq polymerase and de-ionized water.

Initially, a gradient PCR was carried out in a thermal cycler with the following steps: 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, gradient annealing temperature at 55, 55.8, 57.1, 59, 61.2, 63, 64.3 and 65°C for 30 s, extension at 72°C for 1 min and final extension of 4 min at 72°C. The PCR products at different gradients were observed in 2% agarose gel in UV light. Finally, the PCR was carried out at an annealing temperature of 60°C for 30 s keeping other parameters same and the amplicons were separated in a 2% agarose gel via electrophoresis and analyzed in UV light. Annealing temperature 60°C was selected to decrease the chance of non-specific binding of the primers with other DNA sequences. The PCR amplicon was purified using GFX column and sequenced at GCC Biotech, India.

For determination of sensitivity, serial dilutions of *N. meningitidis* cells in 0.5 ml of patient CSF were taken and centrifuged at 16,000 x g for 1 min. The upper viscous CSF was discarded and the pellet was suspended in 0.5 ml of 10 mM Tris and 1 mM EDTA (TE) buffer, pH 8. The bacterial suspension was heated at 95°C for 5 min and centrifuged at 16,000 x g for 1 min. The amount of the genomic DNA (G-DNA) was quantified from the supernatant of each dilution by NanoDrop spectrophotometer and PCR was carried out using different concentrations of genomic DNA as described above. The PCR amplicons were detected in 2% agarose gel to determine the minimum concentration of G-DNA of *N. meningitidis* required for PCR.

Meningitis suspected 25 patient samples obtained from NCDC, Delhi were tested using the present available methods and our 202 bp nspA gene amplicon as a genetic marker. The PCR was also carried out as described above using patient positive sample and 50 ng of G-DNA from *N. meningitidis*, *E. coli*, *S. pyogenes*, *S. pneumoniae*, *S. typhi*, *H. influenzae* and *M. tuberculosis* to check the cross-reactivity of nspA gene amplicon (202 bp) as a genetic marker.

**Results and Discussion**

The gradient annealing temperature PCR showed comparatively less PCR yield at 63, 64.3 and 65°C, while the amount of PCR product remained almost same from 55-61.2°C (Fig. 1). Therefore, 60°C was chosen as standard annealing temperature for the PCR amplification. The PCR product of nspA gene (202 bp) of *N. meningitidis* (Fig. 2) was purified using GFX column and sequenced at TCGA, New Delhi. The 202 bp nspA fragment (amplicon) did not show homology with any other bacterial DNA sequence. Therefore, we developed a quick PCR method (80 min) for detection of *N. meningitidis* directly from patient CSF using nspA gene amplicon as a genetic marker.

The diagnosis of 25 suspected meningitis patients was carried out PCR using 202 bp nspA gene amplicon as a genetic marker, as well as presently available methods (Table 1). Out of the 25 samples, 4
samples were found positive and 1 sample false positive by microscopic, latex agglutination and glucose test, whereas 2 false positive by sucrose test. Similarly, 1 false negative by oxidase and 2 false negative by catalase test were found, while 3 samples were really positive by biosensor and nspA genetic markers (Table 1). The false positive and false negative results might be due to certain limitations of substrate specificity of the test.

For validation of the results, the cross-reactivity of PCR was carried out using G-DNA (50 ng/PCR) of N. meningitidis in CSF (control), E. coli, S. pyogenes, S. pneumoniae, S. typhi, H. influenzae and M. tuberculosis (Table 2). Only controls showed band in 2% agarose gel electrophoresis, corresponding at 202 bp of nspA gene amplicon. No bands were observed in other bacterial (pathogen) samples, because the primers were specific only to strains of N. meningitidis and amplified only specific (202 bp) sequence of nspA gene, but not other genes of possible pathogens, indicating that 202 bp of nspA gene could be used as a specific genetic marker for diagnosis of bacterial meningitis. The present PCR method can detect minimum 10 ng of G-DNA with sharp band directly from CSF of the patients. However, faint bands were observed with 1.0 ng G-DNA of N. meningitidis used in PCR amplification (figure not shown). Thus, lower limit of detection using this method was upto 1 ng G-DNA.

As N. meningitidis bacteria multiply very fast and become double in every 30 min (exponential growth), thus number of bacteria after infection becomes very high in CSF before appearing of symptoms of meningitis (9.3 x 10^{11} CFU in 0.5 ml CSF of patient after 24 h). Mostly, symptoms in patient appear after 48 h of infection and during that period, number of bacteria in CSF becomes very high and sufficient G-DNA is available for diagnosis of the disease. In future, nspA virulent gene based DNA probe could be used for development of DNA biosensor (amperometric) for quick detection of bacterial meningitis. Although DNA biosensors are sensitive, quick and reliable, but they are not cost-effective. Thus, genetic marker based diagnosis may find use where sophisticated instrument facility is not available.

In conclusion, nspA gene amplicon 202 bp could be used as a specific genetic marker for quick detection of human bacterial meningitis caused by N. meningitidis and may be useful in saving life of patients during an outbreak of the disease. Our assay is better than previously described PCR methods due to the specificity and sensitivity of the nspA gene amplicon.

---

**Table 1—Detection of bacterial meningitis of suspected patients using PCR with nspA amplicon as a specific genetic marker and comparison with current available methods**

<table>
<thead>
<tr>
<th>Results</th>
<th>Microscopic²</th>
<th>Enzyme test¹,¹⁴</th>
<th>Latex agglutination³</th>
<th>Biochemical test¹</th>
<th>Biosensor¹²</th>
<th>nspA Amplicon (202 bp) marker control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>04</td>
<td>02</td>
<td>01</td>
<td>04</td>
<td>04</td>
<td>03</td>
</tr>
<tr>
<td>Negative</td>
<td>21</td>
<td>23</td>
<td>24</td>
<td>21</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>False Positive</td>
<td>01</td>
<td>00</td>
<td>00</td>
<td>01</td>
<td>01</td>
<td>00</td>
</tr>
<tr>
<td>False Negative</td>
<td>00</td>
<td>01</td>
<td>02</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
</tbody>
</table>

Gram staining, oxidase and catalase test, latex agglutination and biochemical test were performed as reported earlier¹,³,¹⁴ Omp85 genosensor¹². Sample: N. meningitidis in CSF, Total suspected patient samples: 25.
to sensitivity (low detection limit up to 1 ng G-DNA), specificity of the primers (nspA gene only) and quick detection (80 min), due to smaller size of amplicon used as genetic marker.

Acknowledgement
Authors thank Department of Science and Technology, Govt of India, New Delhi for funding project to molecular biosensor lab to carry out work on meningitis.

References

Table 2—Cross-reactivity test of nspA amplicon (202 bp) as genetic marker with N. meningitidis and other pathogens

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total no.</th>
<th>Present methods of diagnosis</th>
<th>Omp85 Genosensor</th>
<th>nspA gene amplicon as control (202 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram stain</td>
<td>Enzyme test</td>
<td>Latex test</td>
<td>Biochemical test</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>08</td>
</tr>
<tr>
<td>E. coli</td>
<td>04</td>
<td>-</td>
<td>04</td>
<td>01</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>04</td>
<td>04</td>
<td>-</td>
<td>01</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>04</td>
<td>04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. typhi</td>
<td>04</td>
<td>-</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>04</td>
<td>-</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>04</td>
<td>04</td>
<td>-</td>
<td>NA</td>
</tr>
</tbody>
</table>

P = Positive; N = Negative; NA = Not applicable