Neisseria meningitidis causes meningitis and septicaemia and is therefore responsible for considerable morbidity and mortality in both the developed and developing world (Harrison, 1995; Hart & Cuevas, 1997). The serogroups most commonly linked with meningococcal disease (MD) are A, B, C, Y and W135. In the UK, two serogroups are responsible for a major proportion of MD, B and C (Cartwright et al., 2001; Clarke et al., 2001b; Kyaw et al., 2002). Bacterial pathogens with increased virulence and/or transmissibility have highlighted the importance of effective methods of rapid bacterial identification and epidemiological surveillance (Diggle et al., 2001c; Enright et al., 2000; Virji, 1996). Until the introduction of the meningococcal serogroup C conjugate vaccine (MenC), the incidence of confirmed MD continued to increase at a time of raised public and medical awareness and improved laboratory techniques (Clarke & Edwards, 2000; Clarke et al., 2001d; Kaczmarski et al., 1998).

The serogroup C meningococcus is common throughout Western Europe and is generally associated with more severe disease and higher mortality (Connolly & Noah, 1999; Whalen et al., 1995). Due to the rise of serogroup C MD in the UK during the 1990s, the MenC vaccine was implemented in 1999 in conjunction with the UK Departments of Health offering immunization to everyone aged under 18 (Ritchie, 2001; Salisbury, 2001). Since its introduction, there has been a decrease in serogroup C cases (Kyaw et al., 2002). It is possible that there will be more cases of other serogroups due to both selective pressure and the absence in the bacterial population of a significant proportion of serogroup C strains (Clarke & Edwards, 2000; Clarke et al., 2001b; Maiden & Spratt, 1999).

Enhanced surveillance was therefore introduced during the implementation of MenC vaccines (Clarke et al., 1999). The main aims of this surveillance are to improve the ascertainment, investigation and follow-up of cases, to monitor outbreaks and clusters of MD, to monitor the impact of the vaccines on MD and to detect vaccine failures. This includes the need to monitor the phenotypic and genetic characteristics of the infecting organism. However, a large proportion of cases are not culture-confirmed (Clarke et al., 2002), and non-culture methods do not yet provide the required equivalent information. Although assays have now been developed to provide PCR confirmation of disease, followed by serogroup and serosubtype confirmation (Clarke et al., 2001c; Diggle et al., 2001a; Guiver et al., 2000), full sequence type (multilocus sequence typing; MLST) characterization is not yet available.

As MLST is a PCR-based technique, it has the advantage that

Nucleotide sequence-based typing of meningococci directly from clinical samples

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The unpredictable characteristics of meningococcal disease (MD) make outbreaks complicated to monitor and consequently lead to high levels of public anxiety. Traditional molecular techniques have been utilized in order to understand better the epidemiology of MD, but some have disadvantages such as being highly specialized and labour-intensive, with low reproducibility. Some of these problems have been overcome by using multilocus sequence typing (MLST). This technique exploits the unambiguous nature and electronic portability of nucleotide sequencing data for the characterization of micro-organisms. The need for enhanced surveillance of MD after the introduction of serogroup C conjugate vaccines means that it is important to gain typing information from the infecting organism in the absence of a culture isolate. Here, the application of MLST for the laboratory confirmation and characterization of Neisseria meningitidis directly from clinical samples is described. This involved using a newly designed set of primers that were complementary to nucleotide sequences external to the existing MLST primers already in use for culture-based MLST of meningococci. This combination has produced a highly sensitive procedure to allow the efficient genotypic characterization of meningococci directly from clinical samples.

Abbreviations: CSF, cerebrospinal fluid; MD, meningococcal disease; MLST, multilocus sequence typing.
it can be applied directly to culture-negative clinical specimens such as cerebrospinal fluid (CSF) and blood (Enright et al., 2000). We used our experience in developing other non-culture assays for serogrouping (unpublished data) and serosubtyping (Clarke et al., 2001c) in order to develop a nested MLST approach for the laboratory confirmation and characterisation of N. meningitidis directly from clinical samples. MLST provides nucleotide sequence data from seven housekeeping genes and can be useful for public health management (Clarke et al., 2001a; d; Feavers et al., 1999). MLST is based on the well-tested principles of multilocus enzyme electrophoresis (MLEE), but assigns the alleles at each locus directly by nucleotide sequencing, rather than indirectly from the electrophoretic mobilities of their gene products on starch gels.

Clinical samples received at the Scottish Meningococcus and Pneumococcus Reference Laboratory from patients with clinically suspected MD throughout Scotland between September 2001 and May 2002 were tested for the presence of meningococcal DNA using the IS1106 PCR method (Clarke et al., 2001d; Newcombe et al., 1996). Subsequent confirmation of all IS1106 PCR positives was conducted by using the ctrA DEF-PCR method as described previously (Diggle et al., 2001a). Samples that were positive by the IS1106 and ctrA PCR methods were processed for nested MLST. Seven new primer sets were designed that were complementary to nucleotide sequences external to the existing MLST primers already in use for culture-based MLST of meningococci (Table 1). Clinical samples of blood and CSF that were culture negative were used for evaluation of this nested MLST method. A reference laboratory control meningococcal isolate was used as a positive control in order to guarantee that the newly designed primer sets were able to amplify the regions of meningococcal DNA being studied. PCR preparations, reactions and MLST primers for the secondary PCR amplification were as described previously (Clarke et al., 2001a). Solid-phase and liquid-phase PCR product purification, sequence labelling and nucleotide sequence data analysis were all performed as described previously (Clarke & Diggle, 2002; Clarke et al., 2001a; Diggle & Clarke, 2002b). The sensitivity of the assay was also determined by serial dilution.

The meningococcal control culture was positive using the nested MLST approach, indicating that the newly designed primer sets were able to amplify the regions of meningococcal DNA being studied. Nucleotide sequence data also corresponded with that expected from the seven housekeeping genes. The sensitivity testing indicated that the method could detect as few as 10 genome copies ml⁻¹. A total of 701 clinical samples were processed using the IS1106 PCR method, of which 170 were CSF, 51 were plasma, 380 were serum and 100 were whole blood. From these, 10 were positive using the IS1106 and ctrA PCR methods. Six were amplified successfully by nested MLST and nucleotide sequence data were obtained. Presumably, the DNA present in the four other clinical samples was insufficient for amplification by nested MLST. Samples 1–4 were CSF samples corresponding with sequence types (STs) 352, 1161, 1479 and 2031 (Table 2). Sample 5 was a throat swab that was culture negative, and DNA was amplified and sequenced and found to have an ST-238 profile, whilst sample 6 was whole blood and had an ST-11 profile. All these samples were unrelated, sporadic cases (Table 2).

We have therefore described the development of a nested MLST approach to the laboratory confirmation and characterisation of meningococci directly from clinical samples. Along with other non-culture methods for the laboratory confirmation of MD, non-culture MLST will play an important part in the future surveillance of this disease. It is important to note that treatment with antibiotics can successfully limit the onset or decrease the level of disease and is recommended treatment upon immediate suspicion of meningitis (Cartwright et al., 1992). Although this therapy may lead to culture-negative samples, DNA usually remains in a clinical sample taken after the onset of MD and can be amplified by the IS1106 PCR method, and the nested MLST approach was able to confirm all positive samples.

### Table 1. Amplification primers used for primary DNA amplification

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>abZ (OF)</td>
<td>TTCCTTTCGCACTGCGGCGGCT</td>
</tr>
<tr>
<td>abZ (OR)</td>
<td>GCCATCTCCGCTTCTACTGT</td>
</tr>
<tr>
<td>adK (OF)</td>
<td>CCAAGAAGCTGACACAGCT</td>
</tr>
<tr>
<td>adK (OR)</td>
<td>CTTGACGAGGACGATCCTCAT</td>
</tr>
<tr>
<td>aroE (OF)</td>
<td>GGTGATGCGGATGTTGGA</td>
</tr>
<tr>
<td>aroE (OR)</td>
<td>GGTGATGCGGATGTTGGA</td>
</tr>
<tr>
<td>fimC (OF)</td>
<td>CGGCTCTGACGGGCTCCTC</td>
</tr>
<tr>
<td>fimC (OR)</td>
<td>GAGGAAAAAGATTAGGCGGAT</td>
</tr>
<tr>
<td>gdh (OF)</td>
<td>TCACATATCCACGCGCCGT</td>
</tr>
<tr>
<td>gdh (OR)</td>
<td>TTATCTGGGAGGTGCTG</td>
</tr>
<tr>
<td>pshC (OF)</td>
<td>AGCTGAGGCGACGACCTTC</td>
</tr>
<tr>
<td>pshC (OR)</td>
<td>TCAGGATTGCGGTGTGAC</td>
</tr>
<tr>
<td>pgm (OF)</td>
<td>CGGAGCGGGAGATTTTTAGC</td>
</tr>
<tr>
<td>pgm (OR)</td>
<td>GTCTGATGCGGTCGAAAAAC</td>
</tr>
</tbody>
</table>

OF, Outer forward; OR, outer reverse.
Table 2. Non-culture multilocus sequence analysis results for six clinical samples

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Clinical type</th>
<th>Allelic profile*</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CSF</td>
<td>4–10–2–5–8–11–9</td>
<td>352</td>
</tr>
<tr>
<td>2</td>
<td>CSF</td>
<td>4–10–3–4–5–8–11–9</td>
<td>1161</td>
</tr>
<tr>
<td>3</td>
<td>CSF</td>
<td>4–10–3–4–8–8–11–7</td>
<td>1479</td>
</tr>
<tr>
<td>4</td>
<td>CSF</td>
<td>2–6–4–3–9–4–6</td>
<td>2031</td>
</tr>
<tr>
<td>5</td>
<td>Throat swab</td>
<td>7–4–10–5–9–8–11–17</td>
<td>238</td>
</tr>
</tbody>
</table>
| 6       | Whole blood   | 2–3–4–3–8–4–6 | 11| | *Represents the seven different alleles that determine the ST.

therefore be detected and characterized, as demonstrated by this non-culture MLST. Nucleotide sequencing, once an expensive and highly specialized technique, has become more widely available, mainly due to reduced consumable and equipment costs (Diggle & Clarke, 2002a). The availability of MLST data is important as, along with serogroup information, it can be used to monitor capsule switch in meningoocci. Moreover, with porA nucleotide sequence data, which can be gained by culture or non-culture methods, it can be used for public health management during case clusters. MLST data can also be used in order to understand better the population biology of the meningoocci; relationships between strains are apparent by comparing the STs. Databases containing allelic profiles and associated epidemiological data for more than 2000 meningoocci can be accessed at the Neisseria MLST website (http://neisseria.org/mlst/) and provide a single central resource for global epidemiology via the internet. In this way, endemic or epidemic MD can be monitored.

Acknowledgements

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