Short Communication

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Received 19 September 2002

Accepted 25 February 2003

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.

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; Kaczmarek *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 implemen-

ted 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

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 characterization 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 primers 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 and reactions for the primary DNA amplification were

performed in a final volume of 25 µl using 1·1 × Reddymix PCR Master Mix, containing 1·25 U *Taq* DNA polymerase (Abgene), 75 mM Tris/HCl (pH 8·8 at 25 °C), 20 mM (NH₄)₂SO₄, 1·5 mM MgCl₂, 0·01 % (v/v) Tween 20, 0·2 mM each of dATP, dCTP, dGTP and TTP and red dye for gel electrophoresis. For a 25 µl reaction, 20 µl PCR master mix and 1 µl of each MLST primer (Table 1) were added to produce a master-mix volume of 22 µl. Within a refrigerated non-cross-contamination (NCC) 96-well plate, 22 µl master mix was added to appropriate wells with 3 µl DNA preparation, making a final 25 µl reaction mixture. The PCR conditions were designed so that a common amplification method was used for all seven genes to ensure complete and reproducible amplification. The PCR conditions were 95 °C for 2 min followed by 30 cycles at 95 °C for 1 min, 57 °C for 90 s and 72 °C for 2 min and finally 72 °C for 5 min. 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 characterization 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

Table 1. Amplification primers used for primary DNA amplification

Target	Primer sequence (5'–3')
<i>abcZ</i> (OF)	TTCTTTTGCCATCGGCCACGT
<i>abcZ</i> (OR)	GGCATTGCCGTCTGTTTGGT
<i>adk</i> (OF)	CCAAAAAGTTGCCGGCTTG
<i>adk</i> (OR)	CTCAAGCCTCCGACAAACTGT
<i>aroE</i> (OF)	TTCAGACGGCATCGTTCCCAT
<i>aroE</i> (OR)	GCCTGATGGCGTTTGAATGC
<i>fumC</i> (OF)	CCGTCTGAACGCTTTTCAGAC
<i>fumC</i> (OR)	GAGGGAAAGATTAGCGCGGAT
<i>gdh</i> (OF)	TCACATATTCCCACGCGGCTT
<i>gdh</i> (OR)	AATTCTTCGCGCAAGCCTGTG
<i>pdhC</i> (OF)	AGCTACGCCAACAGCAACTTC
<i>pdhC</i> (OR)	TCGGGCATTGCGTTTTCAGAC
<i>pgm</i> (OF)	CGGACGCGGAGATTTTAAAGC
<i>pgm</i> (OR)	GTGCTATGCCGTCTGAAAACC

OF, Outer forward; OR, outer reverse.

Table 2. Non-culture multilocus sequence analysis results for six clinical samples

Isolate	Clinical type	Allelic profile*	ST
1	CSF	4–10–2–5–8–11–9	352
2	CSF	4–10–34–5–38–11–9	1161
3	CSF	4–10–34–8–38–11–7	1479
4	CSF	2–6–4–3–9–4–6	2031
5	Throat swab	7–4–10–15–9–8–11–17	238
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 meningococci. 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 meningococcus; relationships between strains are apparent by comparing the STs. Databases containing allelic profiles and associated epidemiological data for more than 2000 meningococci can be accessed at the Neisseria MLST website (<http://neisseria.org/nm/typing/mlst/>) and provide a single central resource for global epidemiology via the internet. In this way, endemic or epidemic MD can be monitored.

Acknowledgements

This publication made use of the Multi Locus Sequence Typing website (<http://www.mlst.net>) developed by Dr Man-Suen Chan and David Aanensen and funded by the Wellcome Trust. Funding for the robot liquid-handling systems and DNA sequencers was provided by the Meningitis Association (Scotland) and the National Services Division of the Scottish Executive.

References

- Cartwright, K., Reilly, S., White, D. & Stuart, J. (1992). Early treatment with parenteral penicillin in meningococcal disease. *BMJ* **305**, 143–147.
- Cartwright, K., Noah, N. & Peltola, H. (2001). Meningococcal disease in Europe: epidemiology, mortality, and prevention with conjugate vaccines. Report of a European advisory board meeting Vienna, Austria, 6–8 October, 2000. *Vaccine* **19**, 4347–4356.
- Clarke, S. C. & Diggle, M. A. (2002). Automated PCR/sequence template purification. *Mol Biotechnol* **21**, 221–224.
- Clarke, S. C. & Edwards, G. F. (2000). Implications for the serogroup incidence of meningococcal disease after the introduction of the MenC vaccine. *Scott Med J* **45**, 67.
- Clarke, S. C., Christie, P. & Edwards, G. F. (1999). Enhanced surveillance of meningococcal disease in Scotland. *SCIEH Wkly Rep* **33**, 235–236.
- Clarke, S. C., Diggle, M. A. & Edwards, G. F. (2001a). Semiautomation of multilocus sequence typing for the characterization of clinical isolates of *Neisseria meningitidis*. *J Clin Microbiol* **39**, 3066–3071.
- Clarke, S. C., Reid, J., Thom, L., Denham, B. C. & Edwards, G. F. (2001b). Meningococcal disease due to serogroup Y in Scotland, 1992–1999. *Br J Biomed Sci* **58**, 17–19.
- Clarke, S. C., Diggle, M. A. & Edwards, G. F. (2001c). Automated non-culture-based sequence typing of meningococci from body fluids. *Br J Biomed Sci* **58**, 230–234.
- Clarke, S. C., Diggle, M. A., Reid, J. A., Thom, L. & Edwards, G. F. (2001d). Introduction of an automated service for the laboratory confirmation of meningococcal disease in Scotland. *J Clin Pathol* **54**, 556–557.
- Clarke, S. C., Reid, J., Thom, L., Denham, B. C. & Edwards, G. F. (2002). Laboratory confirmation of meningococcal disease in Scotland, 1993–99. *J Clin Pathol* **55**, 32–36.
- Connolly, M. & Noah, N. (1999). Is group C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993–6. European Meningitis Surveillance Group. *Epidemiol Infect* **122**, 41–49.
- Diggle, M. A. & Clarke, S. C. (2002a). What a load of old sequence!!! *J Clin Microbiol* **40**, 2707.
- Diggle, M. A. & Clarke, S. C. (2002b). Rapid assignment of nucleotide sequence data to allele types for multi-locus sequence analysis (MLSA) of bacteria using an adapted database and modified alignment program. *J Mol Microbiol Biotechnol* **4**, 515–517.
- Diggle, M. A., Edwards, G. F. & Clarke, S. C. (2001a). Automation of fluorescence-based PCR for confirmation of meningococcal disease. *J Clin Microbiol* **39**, 4518–4519.
- Diggle, M. A., Edwards, G. F. S. & Clarke, S. C. (2001b). Developments in the diagnosis of meningococcal disease and the characterization of *Neisseria meningitidis*. *Rev Med Microbiol* **12**, 211–217.
- Enright, M. C., Knox, K., Griffiths, D., Crook, D. W. & Spratt, B. G. (2000). Molecular typing of bacteria directly from cerebrospinal fluid. *Eur J Clin Microbiol Infect Dis* **19**, 627–630.
- Feavers, I. M., Gray, S. J., Urwin, R., Russell, J. E., Bygraves, J. A., Kaczmarek, E. B. & Maiden, M. C. (1999). Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. *J Clin Microbiol* **37**, 3883–3887.
- Guiver, M., Borrow, R., Marsh, J., Gray, S. J., Kaczmarek, E. B., Howells, D., Boseley, P. & Fox, A. J. (2000). Evaluation of the Applied Biosystems automated Taqman polymerase chain reaction system for the detection of meningococcal DNA. *FEMS Immunol Med Microbiol* **28**, 173–179.
- Harrison, L. H. (1995). The worldwide prevention of meningococcal infection. Still an elusive goal. *JAMA* **273**, 419–421.
- Hart, C. A. & Cuevas, L. E. (1997). Meningococcal disease in Africa. *Ann Trop Med Parasitol* **91**, 777–785.
- Kaczmarek, E. B., Ragunathan, P. L., Marsh, J., Gray, S. J. & Guiver, M. (1998). Creating a national service for the diagnosis of meningococcal disease by polymerase chain reaction. *Commun Dis Public Health* **1**, 54–56.
- Kyaw, M. H., Clarke, S. C., Christie, P., Jones, I. G. & Campbell, H. (2002). Invasive meningococcal disease in Scotland, 1994 to 1999, with emphasis on group B meningococcal disease. *J Clin Microbiol* **40**, 1834–1837.
- Maiden, M. C. & Spratt, B. G. (1999). Meningococcal conjugate vaccines: new opportunities and new challenges. *Lancet* **354**, 615–616.
- Newcombe, J., Cartwright, K., Palmer, W. H. & McFadden, J. (1996). PCR of peripheral blood for diagnosis of meningococcal disease. *J Clin Microbiol* **34**, 1637–1640.

Ritchie, L. D. (2001). *Meningococcal Group C Immunisation Programme in Scotland: Final Report*. Glasgow: Meningococcal Group C Programme – Implementation Group.

Salisbury, D. (2001). Meningococcal group C vaccine and the United Kingdom experience. *Int J Clin Pract Suppl*, 10–11.

Virji, M. (1996). Meningococcal disease: epidemiology and pathogenesis. *Trends Microbiol* **4**, 466–469.

Whalen, C. M., Hockin, J. C., Ryan, A. & Ashton, F. (1995). The changing epidemiology of invasive meningococcal disease in Canada, 1985 through 1992. Emergence of a virulent clone of *Neisseria meningitidis*. *JAMA* **273**, 390–394.