Short Communication

Automation of a fluorescence-based multiplex PCR for the laboratory confirmation of common bacterial pathogens

Karen Smith,[1] Mathew A. Diggle[1] and Stuart C. Clarke[1,2]

[1] Scottish Meningococcus and Pneumococcus Reference Laboratory, Stobhill Hospital, Glasgow, Scotland, UK

[2] Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK

Correspondence
Stuart C. Clarke
stuart.clarke@northglasgow.scot.nhs.uk

Received 7 August 2003
Accepted 22 October 2003

A fluorescence-based multiplex PCR was automated for the simultaneous detection of *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* in clinical samples from patients with suspected meningitis. Sensitivity of one to two genome copies per 100 μl sample and specificity of 100 % for each organism were shown. Automation of DNA extraction, liquid handling, PCR and analysis are achieved on a single platform, which enables a high throughput and rapid turnaround of clinical samples that, in turn, leads to faster diagnosis. This is ultimately beneficial to the treatment of the patient and for public health management.

Introduction

Bacterial meningitis is a major cause of morbidity and mortality in all societies worldwide. The rapid progression of symptoms and potentially devastating effect of this disease necessitate early recognition and immediate treatment (Rosenstein *et al.*, 2001). *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* are most commonly associated with bacterial meningitis (Peltola, 2000; van Deuren *et al.*, 2000; Koedel *et al.*, 2002). The organism causing disease is traditionally identified after culturing bacteria from a normally sterile body site. Antibiotics are prescribed routinely as part of the pre-hospital management of bacterial meningitis and, subsequently, this may render culture of the causative organism difficult. However, diagnosis of bacterial disease has improved in recent years, due to the routine use of PCR in the clinical laboratory (Clarke, 2002). Bacterial DNA can be extracted from body fluids of patients with suspected meningitis and amplified to a detectable level by PCR. This enables detection of the causative organism by presence of its DNA, rather than from a culture (Corless *et al.*, 2001; Diggle & Clarke, 2002; Smith *et al.*, 2003).

Increasing demand for more sensitive and rapid diagnostic procedures, such as fluorescence-based PCR, has prompted the routine use of automated liquid-handling systems in the clinical laboratory (Clarke, 2002). Automated systems have become more affordable in recent years and also more attractive, because of their ability to process a large number of samples rapidly, with high accuracy and reproducibility. This is invaluable in the clinical laboratory setting, where the rapid and accurate diagnosis of bacterial disease is crucial. We therefore automated a fluorescence-based multiplex PCR for the simultaneous detection of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*.

Abbreviations: CSF, cerebrospinal fluid; SMPRL, Scottish Meningococcus and Pneumococcus Reference Laboratory.

Methods

Bacterial DNA was extracted from clinical samples by using a liquid-handling robot and automated DNA-binding plate system. The robotic system performed all liquid handling; this allowed the rapid extraction of DNA from up to 96 samples, producing a high yield of bacterial DNA for use in PCR. The PCR set-up and cycling protocol were also automated on the liquid-handling robot. The robot was programmed, according to the manufacturer's instructions, to analyse up to 96 samples in one run, allowing a much higher throughput than could be achieved manually. This also reduced the possibility of contamination, as the robot has a non-cross-contamination system.

The assay was validated by using 500 clinical samples that had been submitted to the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) for routine PCR testing. The samples were submitted between September 2002 and January 2003 and consisted of serum ($n = 285$), whole blood/EDTA ($n = 140$), cerebrospinal fluid (CSF) ($n = 72$) and pleural fluid ($n = 1$). Patients' ages ranged from 2 days to 90 years. All samples were subjected to DNA extraction by using the Promega Wizard SV96 purification system (Promega) on a Roboseq 4200 PE liquid-handling robot (MWG Biotech), as described previously (Smith *et al.*, 2003). Oligonucleotide primers were based on previously published *ctrA*, *ply* and *bexA* gene sequences for meningococcal, pneumococcal and *H. influenzae* DNA, respectively (Corless *et al.*, 2001; Diggle *et al.*, 2001) (all oligonucleotide primers and the *ctrA* and *ply* probes were supplied by MWG Biotech; the *bexA* probe was supplied by Biosource International). PCRs were also performed as described previously (Corless *et al.*, 2001; Smith *et al.*, 2003), except that ABsolute

QPCR Mastermix (ABgene) was used in place of Reddymix PCR Mastermix (also ABgene). An integrated BIO-TEK FL600 microplate reader (MWG Biotech) was used to measure fluorescence emitted from each sample. Cut-off levels were calculated for each reaction from the mean negative control and SD between negative controls (Diggle *et al.*, 2001). The SD was used in combination with the mean negative control to calculate a suitable cut-off point. Any value above this level was noted as positive.

Results

Of 500 samples tested, 25 positive cases of meningococcal infection, 20 positive cases of pneumococcal infection and one infection with *H. influenzae* were detected by the fluorescence-based multiplex PCR. Meningococcal isolates were received by the SMPRL for only four of the 25 cases that were detected by multiplex PCR, and only four of the 20 pneumococcal infections detected.

Sensitivity of the fluorescence-based PCR was determined by inoculating 100 µl whole blood with two colonies of *N. meningitidis*, *S. pneumoniae* and *H. influenzae*. A series of tenfold dilutions of the sample was set up and the test was carried out in triplicate. DNA was extracted from 50 µl of each sample in the dilution series by using the Promega Wizard® SV96 purification system. The other 50 µl was cultured on blood or chocolate agar at 37 °C in 5 % CO_2 overnight. Following incubation, colony counts were performed for each agar plate and fluorescence-based PCR was performed on the samples from which DNA was extracted. Sensitivity of the PCR was calculated as the minimum number of colonies that was necessary to give a positive result by fluorescence-based PCR. All three primer sets could detect a mean of one to two genome copies per 100 µl sample. Primers were tested in single reactions and in a multiplex reaction and showed the same sensitivity in each case. The presence of three primer sets in the reaction had no effect on its sensitivity.

The primer sets that were used in the fluorescence-based PCR were also analysed for their specificity. These primers were tested with 3 µl genomic DNA that had been extracted from a heavy growth of culture from bacteria that were most likely to be present in CSF and blood during meningococcal infection. These included oral streptococci, other neisseriae and several other bacteria that are capable of causing septicaemia. Importantly, primers were tested to ensure they amplified the common serogroups and serotypes of *N. meningitidis* and *S. pneumoniae*, respectively, and *H. influenzae* serotypes b and c (Table 1). The three primer sets were highly specific for all strains of *N. meningitidis*, *S. pneumoniae* and *H. influenzae* tested and produced no false-positive results with any other species of *Neisseria*, *Streptococcus* or other pathogenic bacteria that were used.

Table 1. Specificity test results

Organism	No. tested	No. positive (%)	No. negative (%)
Haemophilus influenzae:			
Type a	5		5 (100)
Type b	5	5 (100)	
Type c	5	5 (100)	
Type d	5		5 (100)
Type e	5		5 (100)
Type f	5		5 (100)
Neisseria meningitidis:			
Serogroup A	10	10 (100)	
Serogroup B	10	10 (100)	
Serogroup C	10	10 (100)	
Serogroup W135	10	10 (100)	
Serogroup Y	10	10 (100)	
Neisseria lactamica	4		4 (100)
Neisseria mucosa	4		4 (100)
Neisseria gonorrhoeae	4		4 (100)
Streptococcus pneumoniae	20	20 (100)	
Streptococcus agalactiae	4		4 (100)
Streptococcus oralis	4		4 (100)
Streptococcus mitis	4		4 (100)
Streptococcus parasanguinis	4		4 (100)
Staphylococcus aureus	4		4 (100)
Escherichia coli	4		4 (100)

Discussion

Successful treatment and recovery of individuals who are suffering from bacterial septicaemia or meningitis, as well as effective public health management of contacts, relies upon rapid diagnosis. The development of better methods is therefore important to improve the sensitivity and specificity of such diagnosis. Rapid and sensitive PCR methods have been developed in recent years, but many of them are of low throughput and are not automated. In this study, the entire process, from extraction of bacterial DNA from clinical samples to PCR set-up and cycling protocol, was automated successfully. DNA extraction from up to 96 samples took approximately 50 min. The robot can handle a large number of clinical samples efficiently in a shorter time-scale than could be achieved manually. Liquid-handling technology allows accurate and reproducible protocols to be performed. Previously, meningococcal infections were confirmed in the SMPRL by using a PCR that detected the insertion element IS*1106* (Clarke *et al.*, 2002). The assay was sensitive, but lacked the specificity of the *ctrA* assay. Genetic mobility of the insertion element resulted in its spread to organisms other than *N. meningitidis* (Borrow *et al.*, 1998). *ctrA* provides a more specific alternative target for confirmation of meningococcal infection (Guiver *et al.*, 2000). The multiplex PCR described here detects the three most common pathogenic organisms that cause bacterial meningitis in a single reaction. It is a highly sensitive assay with the ability to detect a mean of one to two genome copy units per 100 µl clinical sample and allows non-culture-based diagnosis of the disease-causing organism within 3 h.

Acknowledgements

This work was presented at the 103rd General Meeting of the American

Society for Microbiology in Washington on 20th May 2003, poster number C-147. Funding for the liquid-handling robot was generously provided by the Meningitis Association (Scotland) and the National Services Division of the Scottish Executive. We would like to thank Wyeth UK for part funding.

References

Borrow, R., Guiver, M., Sadler, F., Kaczmarski, E. B. & Fox, A. J. (1998). False positive diagnosis of meningococcal infection by the IS*1106* PCR ELISA. *FEMS Microbiol Lett* **162**, 215–218.

Clarke, S. C. (2002). Nucleotide sequence-based typing of bacteria and the impact of automation. *Bioessays* **24**, 858–862.

Clarke, S. C., Reid, J., Thom, L., Denham, B. C. & Edwards, G. F. S. (2002). Laboratory confirmation of meningococcal disease in Scotland, 1993–9. *J Clin Pathol* **55**, 32–36.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. & Kaczmarski, E. B. (2001). Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol* **39**, 1553–1558.

Diggle, M. A. & Clarke, S. C. (2002). Semi-automation of the polymerase chain reaction for laboratory confirmation of meningococcal disease. *Br J Biomed Sci* **59**, 137–140.

Diggle, M. A., Edwards, G. F. S. & Clarke, S. C. (2001). Automation of fluorescence-based PCR for confirmation of meningococcal disease. *J Clin Microbiol* **39**, 4518–4519.

Guiver, M., Borrow, R., Marsh, J., Gray, S. J., Kaczmarski, 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.

Koedel, U., Scheld, W. M. & Pfister, H.-W. (2002). Pathogenesis and pathophysiology of pneumococcal meningitis. *Lancet Infect Dis* **2**, 721–736.

Peltola, H. (2000). Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. *Clin Microbiol Rev* **13**, 302–317.

Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T. & Hughes, J. M. (2001). Meningococcal disease. *N Engl J Med* **344**, 1378–1388.

Smith, K., Diggle, M. A. & Clarke, S. C. (2003). Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. *J Clin Microbiol* **41**, 2440–2443.

van Deuren, M., Brandtzaeg, P. & van der Meer, J. W. M. (2000). Update on meningococcal disease with emphasis on pathogenesis and clinical management. *Clin Microbiol Rev* **13**, 144–166.