PCR–Single-Stranded Conformational Polymorphism Analysis for Non-Culture-Based Subtyping of Meningococcal Strains in Clinical Specimens

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Subspecific typing of clinical meningococcal strains is important in the investigation of outbreaks and for disease surveillance. Serogrouping, typing, and subtyping of strains currently require isolation of a meningococcus from one or more clinical specimens. However, the increasing widespread practice of preadmission administration of parenteral antibiotics has resulted in a decrease in the frequency of positive cultures obtained from blood and cerebrospinal fluid. Confirmation of meningococcal disease can be obtained by meningococcus-specific PCR from both cerebrospinal fluid (H. Ni et al., *Lancet* 340:1432–1434, 1992) and peripheral blood (J. Newcombe et al., *J. Clin. Microbiol.* 34:1637–1640, 1996) specimens. However, current PCR protocols do not yield epidemiologically useful typing information. We report here the use of PCR–single-stranded conformational polymorphism (PCR–SSCP) analysis to amplify and type meningococcal DNA present in clinical specimens. PCR–SSCP analysis with the VR1 region of the *Neisseria meningitidis* *porA* gene as the target produced unique banding patterns for each serosubtype. Direct PCR–SSCP of clinical specimens can therefore provide typing data that can be used to investigate the epidemiology of clusters of cases and outbreaks and for disease surveillance in situations in which culture of patient specimens proves negative.

Meningococcal disease remains an important health problem in the United Kingdom and worldwide. Confirmation of the diagnosis of meningococcal disease is becoming increasingly difficult. Meningococcal disease is often first suspected on clinical grounds and is then confirmed by isolation of *Neisseria meningitidis* from blood or cerebrospinal fluid (CSF) or by microscopic detection of gram-positive diplococci in CSF. In untreated patients, meningococci can be recovered from cultures of blood from about 50% of patients with meningococcal disease and from cultures of CSF from about 90% of those patients in whom lumbar puncture is undertaken. However, preadmission antibiotic treatment, which reduces mortality and is recommended by the Chief Medical Officer of the United Kingdom, reduces the chance of a positive blood culture to 5% or less and that of a positive CSF culture to 50% or less (4). Furthermore, recent review of meningococcal disease cases in West Gloucestershire confirms a trend to the less frequent use of lumbar puncture in younger patients. In the absence of a microbial culture, confirmation of the diagnosis can be made by PCR, which can detect the presence of meningococcal DNA in both CSF (9, 12, 18, 21, 22) and blood specimens (19), even after antibiotic treatment. However, present PCR tests do not give information about the subtype of the meningococcal strain present. Meningococci are serogrouped on the basis of variations in the capsular polysaccharide antigen and are serotyped and serosubtyped on the basis of variations in outer membrane protein antigens class 2/3 and class 1, respectively. More detailed characterization of strains

from patients and carriers of the meningococcus is important for the following reasons. (i) Vaccines are available for serogroup A, C, Y, and W135 strains, and it is therefore important to identify outbreaks associated with these serogroups. (ii) Typing and subtyping may be used to demonstrate epidemiological links between case patients and between case patients and carriers in an outbreak situation. (iii) Typing and subtyping are important for monitoring the changing epidemiology of disease; subtyping is especially important because multivalent serogroup B vaccines based on class 1 outer membrane proteins (2, 17, 24, 27) are being evaluated. Serotyping and serosubtyping will be extremely important in a postvaccination era to detect the presence of new variants of class 2/3 and class 1 outer membrane proteins and to monitor strains isolated from patients in whom vaccines have failed. Although a number of PCR-related techniques have been used for subspecific typing of the meningococcus (1, 13, 14, 29), all of these require prior culture of the organism.

PCR–single-stranded conformational polymorphism (PCR–SSCP) technique was developed in order to obtain subspecific typing information on the meningococci present in blood and CSF. A segment of the *porA* gene containing the variable region VR1 was amplified. VR1 is the target for many of the serosubtyping antibodies, and considerable information concerning sequence variation at this locus is available (15, 16, 25). PCR amplification was followed by SSCP analysis, which is capable of highly sensitive detection of point mutations in DNA (7, 10, 26). PCR–SSCP analysis was used to demonstrate both the identities and nonidentities of meningococcal strains from clinical specimens and between clinical specimens and cultured strains.

MATERIALS AND METHODS

Clinical specimens and cultures. Clinical specimens of CSF, blood buffy coat, or serum were obtained from Gloucester, Plymouth, and Hereford (United

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All meningococcal cultures had previously been identified by growth on New York City Media (5) and Gram staining and were typed by the Meningococcal Reference Unit, Manchester Public Health Laboratory, Manchester, United Kingdom. In addition, DNA specimens from meningococcal strains that had previously been subjected to restriction fragment length polymorphism (RFLP) typing by using insertion sequence *IS1106* as the probe (11, 20) were also examined.

PCR sample preparation. DNA was purified from 50- μ l blood buffy coat samples or 50- μ l serum samples by the method of Boom et al. (3). The sample was lysed in 900 μ l of lysis buffer (120 g of guanidinium thiocyanate dissolved in 100 ml of Tris-HCl [pH 6.4], 22 ml of EDTA [pH 8], and 2.6 g of Triton X-100) in the presence of 40 μ l of a diatom (Celite; Aldrich Chemicals) suspension. After a 10-min incubation at room temperature, the diatoms were pelleted. This was followed by washing in 1 ml of wash buffer (120 g of guanidinium thiocyanate dissolved in 100 ml of Tris-HCl [pH 6.4]), ethanol, and acetone. The pellet was dried for 10 min at 56°C before the DNA was eluted from the diatoms by heating at 56°C for 10 min in 30 μ l of Tris-EDTA. The DNA samples were stored at -20°C prior to PCR. CSF samples were boiled prior to amplification. DNA was extracted from meningococcal cultures by standard DNA extraction protocols (23).

PCR-SSCP. For the PCR, 3 μ l of eluent, 2 μ l of CSF, or 1 μ l of diluted culture DNA was used. The DNA was amplified by PCR in a 25- μ l reaction mixture containing 200 μ M (each) dCTP, dATP, and dGTP, 400 μ M dUTP, 1.25 U of *Taq* polymerase (Boehringer Mannheim), 8% glycerol, and 150 ng of primer A/1 (5'-CTTACCGCCCTCGTATTG-3' (positions 77 to 94) and primer A/2 (5'-GCAACGGATACGTCTTG-3' (positions 370 to 353), homologous to targets in the meningococcal *porA* gene (the position numbers refer to those in the published DNA sequence of *porA*) (16). If the samples were to be analyzed by laser densitometry, 0.5 nmol of fluorescein-labelled 12-dUTP (Boehringer Mannheim) was incorporated into the PCR mixture as described previously (28) and the dUTP concentration was adjusted to 80 μ M. The PCR conditions were as follows: 95°C for 2 min for 1 cycle and 63°C for 40 s, 72°C for 1 min, and 95°C for 25 s for 32 cycles. The PCR products were boiled for 10 min in the presence of 95% formamide and rapidly cooled. The products were analyzed either by (i) electrophoresis through a Tris-borate-EDTA (TBE) and 8% acrylamide-bisacrylamide (37.5:1) (National Diagnostics) gel with 10% glycerol, and the bands were visualized by staining with ethidium bromide or (ii) by electrophoresis through a TBE and 8% acrylamide-bisacrylamide gel (37.5:1) with 10% glycerol on the Applied Biosystems (ABI) 373A Automated DNA Sequencer. Each PCR product was analyzed in the presence of ROX 25500 standard size markers (ABI) loaded with the sample in each lane at 500 V, 30 A, and 30 W. Bands were detected by laser densitometry with the Genescan 672 software, version 1.2.2-1 (ABI).

RESULTS

PCR-SSCP analysis was first investigated by using DNA extracted from meningococcal cultures that had previously been serosubtyped. A segment of the *porA* gene including the variable region VR1 was amplified by PCR. SSCP analysis was performed by denaturation of the PCR product followed by polyacrylamide gel electrophoresis and ethidium bromide staining of the bands obtained. The specificities of the PCR primers were determined by amplification of DNA from a range of bacteria (*Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus faecalis*, viridans group streptococci, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria lactamica*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Micrococcus luteus*, *Salmonella typhi*, and *Proteus vulgaris*). All the samples were negative except for those containing *N. meningitidis* and *N. gonorrhoeae* (data not shown).

Fifty-two strains belonging to 10 different serosubtypes and 5 nontypeable strains were investigated. The strains belonged to four serogroups: serogroups A (2 strains), B (40 strains), C (6 strains), and W (3 strains). There was also a nongroupable strain. Within the visual limitations of a polyacrylamide gel, each serosubtype tested was found to generate a distinct SSCP banding pattern, with the same serosubtype giving the same pattern. Figure 1 shows eight strains of five serosubtypes, with each serosubtype showing a different pattern. Nine strains of the same serosubtype, B15:P1.16, from the same region of the

FIG. 1. PCR-SSCP analysis of meningococcal DNA from eight strains of five different serosubtypes. SSCP analysis of PCR products was performed by acrylamide gel electrophoresis and ethidium bromide staining. Lane 1, ϕ X174 *Hae*III DNA molecular weight markers; lanes 2 to 9, *N. meningitidis* strains of serosubtypes P1.6, P1.16, P1.6, P1.6, P1.10, P1.2, P1.9, and P1.9, respectively.

United Kingdom and nine B15:P1.16 strains from four other regions of the United Kingdom were also analyzed. Each strain gave similar SSCP banding patterns (data not shown).

To apply the technique to clinical specimens, it was necessary to increase the sensitivity of detection of the PCR-SSCP product bands. Fluorescein-labelled dUTP was incorporated into the PCR mixture, and after denaturation of the PCR product, polymorphic SSCP bands were detected by polyacrylamide gel electrophoresis and laser densitometry with the Applied Biosystems 373A Automated DNA Sequencer. Each sample was electrophoresed with an internal molecular size marker so that the tracks could be superimposed using Genescan 672 software. The sensitivity of the PCR-SSCP analysis was found to be between 10 and 100 fg of (purified) DNA. PCR-SSCP analysis was performed with 34 DNA samples extracted from microbial cultures of strains with 10 different serosubtype designations, including nontypeable strains. Each of the serosubtypes gave a distinct densitometry trace. Figure 2 shows an example tracing obtained with four strains of different serosubtypes. PCR-SSCP analysis was then performed with a group of meningococcal DNA samples that had previously been subjected to extensive RFLP analysis with a variety of probes, including the insertion sequence *IS1106*. DNA samples from 10 B15:P1.16 strains isolated in 1989 during a prolonged outbreak of meningococcal disease in the area of Gloucester, United Kingdom (6, 11), all gave identical SSCP patterns (Fig. 3). These strains had previously been shown to be identical by RFLP analysis (11, 20). A second group of seven B15:P1.16 strains isolated during the period from 1985 to 1988 from elsewhere in the United Kingdom and previously

FIG. 2. PCR-SSCP analysis of meningococcal DNA extracted from microbial cultures of strains with different serosubtypes by laser densitometry on the ABI 373A Automated DNA Sequencer.

FIG. 3. PCR-SSCP analysis of DNA extracted from microbial isolates, all B15:P1.16, from the Gloucester outbreak on the ABI 373A Automated DNA Sequencer.

subjected to RFLP analysis were also shown to give identical SSCP patterns (data not shown), even though the strains were distinct by RFLP analysis (11, 20). This indicates that SSCP analysis is, as expected, sensitive to sequence variation within the *porA* gene but not to sequence variation at other loci in the meningococcal genome.

PCR-SSCP analysis was next performed directly with DNA extracted from 25 clinical specimens and the corresponding microbial isolates from the same patients. First, we examined eight strains from patients for whom both clinical specimens and matching cultures were available. For each patient, the clinical specimen gave an SSCP tracing identical to that for the microbial isolate. Figure 4 shows a representative example in which the result for DNA from a CSF specimen was compared to the results of a similar analysis with DNA extracted from both blood-derived and CSF-derived microbial cultures of clinical specimens isolated from the same patient. As can be seen, densitometry traces that could be superimposed were obtained. Seven clinical specimens from epidemiologically unlinked patients from whom microbial isolates were obtained and subsequently typed as the same serosubtype were next examined. Figure 5 shows a representative example in which three clinical specimens (one serum specimen and two CSF specimens) that each yielded P1.16,7 serosubtype isolates were analyzed. Similar densitometry traces were obtained, although minor differences were occasionally found. For example, in Fig. 5, the single serum sample gave an extra peak that was not found for either of the other two samples. This is likely due to

FIG. 4. PCR-SSCP analysis of DNA extracted from microbial cultures and a clinical sample (CSF) obtained from the same patient. PCR products were detected by laser densitometry on the ABI 373A Automated DNA Sequencer. The *N. meningitidis* strain isolated from this patient was typed as a C2a:NT.

FIG. 5. PCR-SSCP analysis of DNA extracted from clinical specimens from patients from whom *N. meningitidis* strains, each of serosubtype P1.16,7, were isolated.

subspecific sequence variation within the P1.16,7 serosubtype (8, 25). Lastly, we examined DNA extracted from 14 clinical specimens from patients from whom we obtained microbial isolates with different serosubtypes, including three nontypeable strains. Distinct SSCP patterns were obtained. A representative sample is presented in Fig. 6.

DISCUSSION

The rapidly increasing frequency of culture-negative meningococcal disease has made epidemiological surveillance and management of clusters of cases and outbreaks more difficult. PCR may be used to confirm a diagnosis of meningococcal disease in many of these patients, but present PCR techniques do not yield epidemiologically useful data. This study demonstrates that PCR-SSCP analysis may be used to obtain epidemiological data directly from clinical specimens, in the absence of positive cultures. For this study the *porA* gene encoding the serosubtyping class 1 antigen was targeted, since extensive DNA sequence information is available for this gene. The gene has two variable regions (VR1 and VR2) which encode subspecific regions of the *porA* protein that are recognized by monoclonal antibodies. Monoclonal antibodies recognizing the different serosubtypes may recognize either VR1 or VR2. Amplification of only one of these regions was chosen for PCR-SSCP analysis, because it has already been demonstrated that even with serosubtypes for which monoclonal antibodies recognize one of the two variable regions, linked subtype-specific sequence variation may be found in the other variable region.

FIG. 6. PCR-SSCP analysis of DNA extracted from CSF taken from patients from whom *N. meningitidis* strains of four different serosubtypes were isolated.

However, although the data indicate that PCR-SSCP analysis may be used to obtain serosubtyping information on *N. meningitidis* strains directly from clinical specimens, the present study has been restricted to an investigation of whether the technique can be used to demonstrate either the identities or the nonidentities of strains present in clinical specimens when culture-based methods are unavailable. This information may be vital in potential outbreak situations. If a cluster of patients is infected with identical strains, active transmission of a virulent meningococcal strain may be occurring within the community and specific control measures such as carrier screening and community-wide prophylaxis may need to be undertaken. On the other hand, if patients are found to be harboring distinct strains, an outbreak situation is much less likely and control measures may be relaxed. The data presented in Fig. 4, 5, and 6 demonstrate that, in the absence of microbial cultures, PCR-SSCP analysis may be used to determine whether the *N. meningitidis* strains (including nontypeable strains) present in clinical samples from two or more patients are distinct strains.

Although the data are insufficient to establish a typing system for *N. meningitidis* based on PCR-SSCP analysis, the data do indicate that this may be possible. Phase 2 trials of multivalent serogroup B vaccines based on the class I outer membrane protein (2, 17, 24, 27) (the target of both serosubtyping and the PCR-SSCP analysis) are ongoing. However, if a multivalent vaccine based on the class I protein is to be successful, it will have to be designed to protect individuals against the most prevalent serosubtypes responsible for causing meningococcal disease. Current typing based on microbial culture will inevitably miss a substantial proportion of cases and will therefore report incomplete data for serosubtype prevalence. PCR-SSCP analysis may be used to obtain serosubtype-specific information on culture-negative patients and provide a more complete picture of meningococcal disease epidemiology. If, in subsequent years, a multivalent vaccine is introduced, then it will similarly be essential to monitor vaccine failures in order to compare the serosubtype prevalence of strains isolated from patients with the composition of strains in the vaccine. PCR-SSCP analysis may again be invaluable for obtaining serosubtype-specific information on the culture-negative patients.

The present study is limited to examination of the locus responsible for serosubtyping. However, we are investigating application of the technique to genetic loci coding for serotyping and serogrouping antigens. Obtaining information on meningococcal serogroups by PCR-SSCP analysis would have direct application in controlling outbreaks, since identification of group A or C strains would permit the prophylactic use of vaccines.

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