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13. ABSTRACT (Maximum 200 words)
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A rapid and sensitive PCR strategy employed for amplification and sequencing of porA from a single colony-forming unit of Neisseria meningitidis

(Meningococcus; class 1 porin; cerebrospinal fluid; automated sequencing; single-copy amplification; vaccines; clinical diagnosis)

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SUMMARY

The predicted amino acid sequence was determined for the class-1 outer membrane protein, PorA, from a B:15:P1.7,3 strain of Neisseria meningitidis that is currently causing an epidemic of meningitis in Northern Chile. The P1.7,3 PorA showed a unique sequence in the exposed loop 4 of the putative porin structure that is different from all the reported PorA sequences. Based on the nucleotide (nt) sequence of the P1.7,3 porA, we designed two sets of PCR (polymerase chain reaction) primers that specifically amplified porA from any N. meningitidis strain, and a third set of primers that amplified porA only from the P1.7,3 strain. Using these primers, we developed a sensitive double hot-start nested PCR (HNPCR) strategy that could amplify porA and generate nt sequence from as low as a single colony-forming unit. This strategy consisted of three phases of PCR. The first two phases were designed to generate amplified target DNA that could be directly visualized by ethidium bromide staining starting from one to two molecules of Neisseria genome. The third phase was designed to generate a sequence of several hundred nt directly from the amplified DNA. A number of culture-negative cerebrospinal fluid samples from individuals suspected of meningitis during a vaccine trial were analyzed by this strategy to obtain more accurate information on the actual number of cases that occurred in the study and the non-study populations. The basic HNPCR strategy described here could be applied to amplify and sequence target DNAs from any low-copy-number biological sample.

INTRODUCTION

The causative agent of meningococcal meningitis is Neisseria meningitidis, an aerobic Gram-negative diplococcus. The species is divided into serogroups that are defined on the basis of the capsular polysaccharide. A total of twelve serogroups are currently recognized, but most systemic disease is caused by serogroups A, B and C. Effective vaccines based on the capsular polysaccharides are available for groups A, C, Y and W135, but vaccine development for group B is still underway. Most group-B vaccines that are under evaluation are based on the prion...
pical outer membrane proteins (OMPs), since the

most published reports, it was necessary to resort to
Southern hybridization of the amplified DNA with a
specific \(^{32}\text{P}\)-labeled probe in order to determine whether
or not the target DNA was amplified (Li et al., 1991;
Varas et al., 1991). Except for one report (Mullis, 1991),
the problems associated with the amplification and
sequencing from one or few molecules of DNA have not
been carefully investigated, although such an analysis will
have far-reaching applications in many fields of biology.
In this paper, we addressed the question of why it is
problematic to amplify target DNA from a low-copy-
number sample, and developed a double hot-start nested
PCR (HNPCR) strategy that could overcome the problems,
and not only amplify DNA but also generate DNA
sequence directly from as low as a single colony forming
unit (cfu).

Although other investigators have used PCR to am-
plify unique meningococcal DNA sequences from cells in
CSF (Kristiansen et al., 1991; Ni et al., 1992), we selected
the \(\text{por}A\) gene of \(N.\ meningitidis\) as the target gene for
developing the PCR strategy because it was thought to
be a species-specific gene, and its product, the class-I
OMP, appears to play an important role in human im-
munity to group-B disease (Zollinger, 1992). We show
that the HNPCR strategy can be successfully used to
generate nt sequence of \(\text{por}A\) from frozen CSF samples
that were not only culture-negative, but were also nega-
tive for \(\text{por}A\) amplification by the standard PCR proto-
cols. We believe that this strategy would serve as a general
technique to amplify and sequence any target DNA, in-
fected or otherwise, from a low-copy-number sample.

RESULTS AND DISCUSSION

(a) Selection of \(\text{por}A\) target

The deduced aa sequence and the predicted structure of
the class-1 OMP of \(N.\ meningitidis\) strain 8529 are
shown in Fig. 1. This strain is the prototype strain for
subtype P1.3 and is representative of the strains causing
an ongoing epidemic in Northern Chile. The P1.3 subtype
was first identified and characterized on strains isolated
from the Chilean epidemic and has only infrequently been
isolated in other countries. Since we were using this strain
as a vaccine strain, and the class-1 protein was considered
an important component of the vaccine, we wanted to
fully characterize this protein and be able to genetically
manipulate it for purposes of vaccine production and
evaluation. Comparison of this sequence with other pub-
lished \(\text{por}A\) sequences (Barlow et al., 1989; Maiden et al.,
1991; Van der Ley et al., 1991) resulted in the prediction
that the variable region of loop 1 contained the epitope
for subtype P1.7 [this epitope has recently been described
as a 'masked' P1.7 due to a specific 9-bp deletion downstream from this region (Wedge et al., 1993)), and the variable region of loop 4 contained the epitope for subtype P1.3 (the unique aa are shown in bold letters in Fig. 1). Peptides corresponding to the unique regions were synthesized, and were tested for binding to P1.7- or P1.3-specific monoclonal antibodies by ELISA and by Geysen pin techniques (Geysen et al., 1984). The P1.7-specific mAb specifically bound to the loop-1 peptide (aa 18–40) at a serum dilution of 1:204,800, but did not bind to either the loop-4 peptide (aa 170–192) or the outer membrane complex in which the epitope is masked. We confirmed the P1.7-specific epitope to the peptide sequence SGQ (aa 32–34) by Geysen pin analysis (McGuinness et al., 1990). Similarly, the P1.3-specific mAb specifically bound to the loop-4 peptide at a serum dilution of 1:102,400 and also to outer membrane complex at a serum dilution of 1:819,200, but not to the loop-1 peptide. The P1.3-specific epitope was further mapped to the peptide sequence ANGANNTI (aa 177–185) by Geysen pin analysis. These direct binding studies clearly identified the P1.7- and P1.3-specific epitopes in loops 1 and 4, and are consistent with the above predictions that are based on nt sequence analysis.

(b) Design of primers for specific amplification of porA

To identify unique regions in porA, we first compared the porA subtype P1.7,3 sequence determined in this study with the published sequences of porA from other subtypes, and also that of porB. Based on these comparisons, two sets of primers that are complementary to the termini of porA were chosen for PCR (Table 1). These primers were then tested for their specificity using a number of strains of Neisseria species and Branhamella catarrhalis that often exist as normal flora in throat cultures. Escherichia coli and Haemophilus influenzae, both of which are known to cause meningitis, were also included in these experiments. The outermost primers p1 and p2, as shown in Fig. 2A, were found to yield a highly specific 1.1-kb product only with N. meningitidis P1.7,3 (lane 3) and other subtypes of N. meningitidis (Fig. 2B and data not shown), but not with either the commensal Neisseria strains (lanes 4–9), or with H. influenzae or E. coli (lanes 1 and 2). Similarly, the inner nested primers p3 and p4 also specifically amplified a 1-kb porA product only from the N. meningitidis strains (lanes 3–9, Fig. 2B), but not with the commensal strains (lanes 10–13, Fig. 2B, and data not shown). It should be mentioned that another nested primer, covering nt 970–995, was less specific as it occasionally amplified porA from commensal strains (data not shown). This was probably because this primer corresponded to an intramembrane region that is conserved among Neisseria porins.

Fig. 1. Deduced aa sequence of PorA P1.7,3 protein arranged to fit into the simplified unrolled β-barrel structural model for porins. Bold letters within the boxes represent the aa that are unique to the P1.7,3 epidemic strain. The regions that correspond to the nt sequence of primers used for PCR and DNA sequencing are marked with arrows. The aa sequence was deduced from the nt sequence of porA P1.7,3 of the epidemic strain. To determine the nt sequence of porA P1.7,3, two sets of primers (pB and pC, pA and pD, Table I) were synthesized from the published sequence of N. meningitidis subtype P1.16 porA (Barlow et al., 1989). These primer sequences are not specific to the P1.16 subtype, but represent sequences that are conserved among the porA subtypes. A unique 1.2-kb fragment was amplified using pB and pC; pC allowed amplification of porA beyond the stop codon thereby generating the complete sequence of P1.7,3 porA at the 3'-end. For the 5'-end, however, we chose to use pB that is part of the sequence coding for the signal peptide portion of PorA P1.16 because a primer upstream from the 5'-end was not highly specific in generating a unique product. Amplification with pB and pC therefore resulted in the generation of a unique product that codes for the complete mature P1.7,3 PorA. The DNA amplified in two independent PCRs was cloned into M13mpl8 and M13mpl9 vectors, and was sequenced using Sequenase version-2.0 (US Biochemical, Cleveland, OH, USA). The porA sequence in loops 1, 4 and 5 (see below) was reconfirmed by comparing it with that generated by direct sequencing of the PCR-amplified DNA. The sequence was entered into GenBank, and can be accessed using accession No. L02929. The aa sequence was deduced from this nt sequence, and was fit into the sequence to this simplified porin structural model with the aid of the GCG program (Devereux et al., 1984). The primary criteria used in fitting the sequence to this simplified porin structural model were threefold: (i) placing conserved sequences with alternating hydrophilic-hydrophobic residues within the membrane; (ii) requiring short turns within the cytoplasm; and (iii) placing variable, hydrophilic sequences purported to be antigenic in extended loops on the outside surface. The presence of Tyr and Phe residues aligned along the upper and lower boundaries of the membrane as was found in the X-ray crystallographic analyses of the porin of Rhodobacter capsulatus (Weiss et al., 1991) and the porins OmpF and PhoE of E. coli (Cowan et al., 1992) further supported the model shown in the figure. Consistent with the published data, the regions of the P1.7,3 PorA that showed clear differences from the other PorA types were found at the tips of the exposed loops 1 and 4 as shown in bold face within the boxes in the figure. The sources of mAb used for epitope mapping studies are as follows. The mAb 12-1-P1.3 was produced in this laboratory by procedures previously described (Zollinger et al., 1984). The mAb were produced using spleen cells from mice immunized with whole organisms or with outer membrane vesicles. The mAb specific for P1.7 (MN14C11.6) was provided by Dr. Jan T. Poolman, Bilthoven, The Netherlands.
TABLE 1
Primers used in PCR and direct sequencing

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Description</th>
<th>Nucleotide location on P1.3 DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pA</td>
<td>CGT ATC GGG TGT TTG CC</td>
<td>Upstream</td>
</tr>
<tr>
<td>pB</td>
<td>GCC CTC GTA TTG TCC GCA</td>
<td>Leader</td>
</tr>
<tr>
<td>pC</td>
<td>GGG CTG AAG CAG ATT GG</td>
<td>Downstream</td>
</tr>
<tr>
<td>pD</td>
<td>GCC GAT GCC GGT ATT GCG</td>
<td>Inner reverse</td>
</tr>
<tr>
<td>Amplification primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p1</td>
<td>GCG GCC GTT GCC GAT GTC AGC C</td>
<td>Outer forward</td>
</tr>
<tr>
<td>p2</td>
<td>GCG GCA TTA ATT TGA GTG TAG TTG CC</td>
<td>Outer reverse</td>
</tr>
<tr>
<td>p3</td>
<td>CAA AGC CCG GTG GGA AG</td>
<td>Inner forward</td>
</tr>
<tr>
<td>p4</td>
<td>GAT GCT AGC TGG TAT TTC CGC C</td>
<td>Inner reverse</td>
</tr>
<tr>
<td>p5</td>
<td>GTG GAG CGA GCG GTC AG</td>
<td>P1.7-specific forward</td>
</tr>
<tr>
<td>p6</td>
<td>ATT AGC ACC ATT AGC AAG AG</td>
<td>P1.7-specific reverse</td>
</tr>
<tr>
<td>Sequencing primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pl</td>
<td>CAC TCC CCT TAA AGC CGA T</td>
<td>Downstream loop 1</td>
</tr>
<tr>
<td>pIll</td>
<td>CTC AGA CCG GCA TAA TAC A</td>
<td>Downstream loop 4</td>
</tr>
<tr>
<td>pIll</td>
<td>CCC ACA TTG GCG TGT CTC GC</td>
<td>Downstream loop 4</td>
</tr>
</tbody>
</table>

*Primer sequences pA-pD are from the porA P1.16 sequence of Barlow et al. (1989). Primer sequences pl-p6 and p1-pIll are from the porA sequence of 8529, the prototype P1.3 strain (this work).

A third set of nested primers, p5 and p6, were then designed which were specific for porA, subtype P1.7,3. These subtype-specific primers were required for identification of the epidemic subtype of meningococci in Iquique, Chile. These primers were designed in such a way that they are complementary to the unique variable regions in loop 1 and loop 4 of the epidemic strain (see Fig. 1). To test the specificity of these primers, the 1.1-kb porA products amplified from eight well-characterized subtypes and two non-subtypable strains were used for a second amplification by the subtype-specific innermost primers p5 and p6 (Fig 2C). A unique 500-bp product could be amplified only from the type P1.7,3 template (lane 4), whereas the porA of other subtypes did not serve as a target for amplification by these primers (lanes 1-3 and lanes 5-10).

It should be pointed out that all the above PCR experiments were done using an excess of template DNA (approx. 10^6 copies of genome per reaction) in order to rigorously establish the specificity of the primers. The copy number used in these experiments is far greater than what one would expect to encounter in a clinical sample.

(c) A PCR strategy to amplify porA from a single cfu of N. meningitidis

One of the challenging problems in the diagnosis of meningococcal meningitis is the detection of N. meningitidis in clinical samples from suspicious cases that are culture-negative, and therefore contain a very low copy number of target DNA. We sought a sensitive PCR strategy that would have the following features, and would be able to definitively establish the status of every suspicious case of meningitis: (i) it should amplify porA from the theoretical limit of one cfu of N. meningitidis per sample, (ii) the amplified DNA should be visible as a unique EtBr-stained DNA band upon agarose gel electrophoresis; (iii) it should amplify porA from a crude clinical sample such as a CSF of an infected individual, and (iv) it should be compatible with the rapid PDS strategy that we developed recently (Rao and Saunders, 1992), to generate the nt sequence of the important variable regions.

Our main focus was to address the first two objectives since reports on amplification of target DNA from less than ten copies of the genome often failed to generate a unique product (Varas et al., 1991). The amplified DNA usually migrated as a smear. Consequently, Southern blotting and hybridization with a specific probe were required to confirm the presence of target DNA. Indeed, many of our initial attempts to amplify porA from the CSF of patients suspected of having meningococcal infections led to the appearance of a smear rather than a unique product (data not shown). We hypothesized that the main contributing factor for the DNA smear is mis-
priming at non-target sites due to short stretches of homology between the genomic DNA and the 3'-end of the primer (a schematic representation is shown in Fig. 3). Therefore, after the first few cycles of PCR, the target DNA will constitute only a minor component of the amplified DNA, whereas the major fraction is expected to be the misprimed nonspecific DNA thereby generating a DNA smear (Fig. 3). We tested a number of strategies to overcome this problem, and developed a HNPCR strategy to specifically amplify porA from one or a few copies of \textit{N. meningitidis} genome (see legend to Fig. 4 for experimental details). The basic premise of these experiments was as follows: even though the amplified DNA after the first phase PCR is predicted to have a mixture of target DNA and non-target DNAs of random lengths, it should be possible to selectively amplify only the target DNA from this heterogenous mixture by performing a second phase PCR using an inner set of nested primers (Fig. 3). Since the target DNA after first phase PCR will be at a sufficiently high copy number, and since the probability of having a sequence that is complementary to the inner nested primers on both ends of the non-target DNA will be infinitesimally small, we expected that the target DNA now should be amplified efficiently to generate a unique product (Fig. 3). In the third and final step, the amplified DNA will be sequenced by asymmetric PCR using the PDS strategy we developed earlier (Rao and Saunders, 1992). A number of variations of this basic approach were tested using defined samples containing 0.25–40 cfu of \textit{N. meningitidis}. The HNPCR strategy consistently generated a unique product that could be visualized simply by

Fig. 2. Specific amplification of porA from \textit{N. meningitidis} by three sets of primers. The sources of strains used in these experiments are as follows. \textit{N. meningitidis} strains 8529 (prototype of subtype P1.3), 99M, and 1901 were from the culture collection of the Walter Reed Army Institute of Research. Prototype strains M992, M982, S3032, M981, M136, and S3446 were obtained from Dr. Carl Frasch, Bethesda, MD: strain H355 was obtained from Dr. L. Oddvar Froholm, Oslo, Norway. The commensal \textit{Neisseria, B. catarrhalis} and \textit{E. coli} strains were from the ATCC, Rockville, MD. The \textit{H. influenzae} strain was provided by Dr. Arthur Branstrom, Washington, DC. The primers required for PCRs were synthesized using an ABI Model-391 PCR-mate oligodeoxyribonucleotide synthesizer, and were purified by gel filtration using PD-10 columns (Pharmacia-LKB) (Table 1). An isolated colony of \textit{N. meningitidis} or other appropriate strain was picked and resuspended in 1 ml Gey's balanced salt solution containing 0.2% gelatin. An aliquot of this suspension, containing approximately 10⁶ organisms, was heated at 95°C for 10 min, cooled quickly on ice, and the PCR components (200 μM of each dNTP/50 pmol of each primer/2.5 units of Taq polymerase) were added directly to this crude sample to a final volume of 100 μl per reaction. PCRs were done in a Perkin-Elmer thermal cycler (N801-0150) for 30 cycles. Each cycle consisted of denaturation of DNA at 95°C for 1 min, annealing for 1 min either at 50°C (in the case of primer sets p3 and p4, and p5 and p6) or 70°C (in the case of primers p1 and p2), and extension at 70°C for 2 min. (Panel A) The outermost primers p1 and p2 amplified the 1.1-kb porA only from \textit{N. meningitidis}. The bacterial strains used were as follows: lane 1. \textit{H. influenzae}; lane 2. \textit{E. coli}; lane 3. \textit{N. meningitidis}; lane 4. \textit{N. perflava}; lane 5. \textit{N. lactamica}; lane 6. \textit{N. flavescens}; lane 7. \textit{N. flavescens}; lane 8. \textit{N. lactamica}; lane 10. \textit{B. catarrhalis}. (Panel B) The inner nested primers p3 and p4 amplified the 1-kb fragment from all \textit{N. meningitidis} subtypes tested. The bacterial strains used were as follows: lane 1. \textit{E. coli}; lane 2. \textit{H. influenzae}; lane 3. M992 (P1.1); lane 4. 99M (P1.2); lane 5. S3032 (P1.3); lane 6. 1901 (P1.6); lane 7. M982 (P1.9); lane 8. S3446 (P1.15); lane 9. S3032 (P1.12,16); lane 10. \textit{N. lactamica}; lane 11. \textit{N. polysaccharae}; lane 12. \textit{N. subflava}; lane 13. \textit{B. catarrhalis}; lane 14. reagent blank. (Panel C) The innermost nested primers p5 and p6 amplified only the subtype P1.7.3-specific 500-bp fragment from an excess of \textit{por A} template from each strain. The subtypes used were as follows: lane 1. M992 (P1.1); lane 2. 99M (P1.2); lane 3. 1901 (P1.6); lane 4. 8529 (P1.7.3); lane 5. M982 (P1.9); lane 6. S3446 (P1.15); lane 7. S3032 (P1.12,16); lane 8. M981 (non-typable); lane 9. M136 (non-typable); lane 10. S3446 (P1.14); lane 11. reagent blank. Lane S represents \textit{HaeIII}-digested 4X174 DNA as size standards; the sizes of the four bands from the top are 1353, 1078, 872 and 603 bp, respectively. The samples were electro-phoresed on a 1.2% agarose gel and stained with EtBr.
CSF with 1-10 copies of Neisseria genome

First phase PCR
(20 μM dNTPs
20 pmol primers)

Nested PCR
(20 μM dNTPs
20 pmol primers)

Asymmetric PCR
(5 μM dNTPs
ddNTPs
32p-primer)

Electrophoresis and Autoradiography

(d) Amplification of porA from CSF samples

The strategy was then tested by amplifying porA from the CSF of patients suspected of having meningococcal meningitis. The cases had been rated by physicians as suspicious, moderately suspicious, or highly suspicious, based on the clinical features associated with these patients. These samples were frozen for two years at −70°C, and had given a negative result by the culture and Gram-stain techniques, and also by standard PCR protocols (Arnheim and Erlich, 1992). These samples contained either no meningococci or a minimal number of nonviable organisms, and therefore provided an ideal test of this strategy to analyze porA in clinical samples. In order to rigorously test the strategy, these experiments, including the positive and negative controls, were done in a blinded manner. The first set of PCR cycles were done using primers p1 and p2, and the second set was done either with primers p3 and p4 (Fig. 5A) or with the subtype-specific primers p5 and p6 (Fig. 5B). The data in Fig. 5A show that a DNA band of the expected 1-kb size was amplified from one out of three suspicious cases (lanes 3–5), one out of two moderately suspicious cases (lanes 6–7), and two out of three highly suspicious cases (lanes 8–10). When the second set of PCR cycles was done using the subtype-specific primers p5 and p6, all but one of the positive samples (lane 5) showed the unique 500-bp product of the P1.7,3 strain.

(e) Determination of nt sequence of the DNA amplified from CSF samples

In the context of our vaccine efficacy studies, it is important to be able to determine the nt sequence in the variable regions of porA directly from the PCR products. This would allow us to quickly confirm the PCR data, determine the sequence of variable regions that were different from the P1.7,3 prototype strain, and detect any microheterogeneity in sequence that could affect recognition of PorA by vaccine-induced antibodies. An aliquot of the PCR mixture following the second phase PCR, without any purification of template, was directly used for sequencing by a third phase asymmetric PCR in the presence of a single 32P-labeled primer (Rao and
contamination. The reagents were kept in an isolated area, and were carefully taken to prevent any external contamination. This was particularly important during the transfer of samples from the first phase PCR mixture and conditions for hot-start for the nested PCR were exactly as in the first set of PCR cycles, and primers p3 and p4 were used in the second set of PCR cycles. (Panel B) Primers p1 and p2 were used in the first set of PCR cycles, and primers p5 and p6 were used in the second set of PCR cycles. Lane 1, a known positive CSF sample that showed a high titer of meningococci by culturing; lane 2, a known negative; lanes 3–5, suspicious cases; lanes 6 and 7, moderately suspicious cases; lanes 8–10, highly suspicious cases; lane S represents HaeIII-digested φX174 DNA as size standards. The samples were electrophoresed on a 1.2% agarose gel and stained with EtBr.

Saunders, 1992). The sequence ladders of the variable regions of porA amplified from the CSF's are shown in Fig. 6. Amplified products that were PCR positive for subtype P1.7,3 (as shown in Fig. 5B), as expected, gave a sequence that matched perfectly with both loop 1 and loop 4 sequences shown in Fig. 1 (panels of Case 14 in Fig. 6). The single P1.7,3-negative case in the above experiment (lane 5, Fig. 5B) was also confirmed to be negative by DNA sequencing (panels of Case 198 in Fig. 6). The nt sequence of this strain was found to match perfectly with the sequence of variable loops 1 and 4 of strain.
consistent with the other published sequences (Barlow et al., 1989; Maiden et al., 1991; Van der Ley et al., 1991), but is unique in the variable region of loop 4, which we have shown to contain the subtype P1.3 epitope. Since the P1.7,3 subtype has been responsible for over 90% of the group-B disease in Northern Chile, this sequence information is critical for the development of the HNPCR strategy as well as the development and evaluation of potential vaccines for use in that area.

(2) We selected three sets of primers that are highly specific for \textit{N. meningitidis}. Of these, two sets of primers, p1 and p2, p3 and p4, specifically amplified a 1.1-kb and a 1-kb product of \textit{porA}, respectively, whereas the third set of primers, p5 and p6, specifically amplified the 0.5-kb size subtype P1.7,3-specific \textit{porA} product. Some strains of \textit{N. meningitidis} do not express any detectable PorA and are nontypable. Several of these were included in panels testing the primers. All were positive for \textit{porA} and yielded the predicted product with the exception of one isolate which was found to have a deletion of the gene (data not shown). \textit{N. gonorrhoeae}, which appears to harbor a silent \textit{porA} gene, however showed amplification of a 1.1-kb product with the outer primers p1 and p2, and a 0.6-kb product with inner nested primers p3 and p4, suggesting a potentially rearranged \textit{porA} sequence in this organism (data not shown). Barlow et al. (1989) previously suggested the presence of a \textit{porA}-related sequence on the basis of Northern blot analysis. This should not however affect the specificity of the assay or its usefulness in routine testing of clinical samples because the 0.6-kb product generated with p3 and p4 from \textit{N. gonorrhoeae} could be easily distinguished from the 1-kb size \textit{N. meningitidis} \textit{porA} product, and also \textit{N. gonorrhoeae} is unlikely to be present in the CSF samples. Determination of the subtype specificity of \textit{N. meningitidis} has been very useful from the point of view of vaccine efficacy evaluation. This is true because the vaccine is considered to be primarily a subtype-specific vaccine and may not protect against heterologous subtypes. The subtype-specific primers p5 and p6 could be simply replaced with another pair of subtype-specific primers in a different epidemiological setting, or if multiple subtypes are causing disease, a panel of subtype-specific primers could be used to determine the subtype of the isolates.

(3) The problems associated with amplification of DNA from low-copy-number samples have not been fully investigated. From this study, it appears that minimizing the mispriming events, particularly those occurring during the initial cycles of PCR, played a critical role in amplifying a unique DNA fragment from a low-copy-number sample. We believe that mispriming is primarily due to two reasons. First, most protocols recommend the following sequence of steps for standard PCR: (i) boiling the complete PCR mixture, (ii) cooling the samples imme-

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**Fig. 6. Determination of nt sequence of \textit{porA} amplified from CSF samples.** The amplified \textit{porA} was directly sequenced using the PCR-directed sequencing (PDS) strategy described previously (Rao and Saunders, 1992). A 5 µl aliquot of the PCR mixture as in Fig. 5A was mixed with 5 µM each of four dNTPs, 30 pmol 32P-labeled primer, either 100 µM ddGTP, or 750 µM ddATP, or 750 µM ddTTP, or 1 mM ddCTP, 125 µM Mn2+ and 2.5 units of Taq polymerase. PCR cycling was done according to the conditions described earlier (Rao and Saunders, 1992). The labeled products were resolved by electrophoresis on a sequencing gel containing 6% polyacrylamide and 8 M urea. The gel was dried and autoradiographed by exposing to an X-OMAT AR (Kodak) film. Since the variable loops 1 and 4 are of most interest in determining the type specificity, sequencing was done using primers pl, p11 or p1II (Table 1) to cover these regions. (A) Sequence ladders were generated using loop-1 primer, pl. Note a 9-nt and a 3-nt insertion (bracketed with arrows) in Case 198 when compared to Case 14. (B) Sequence ladders were generated using loop-4 primer, p11. Note that the sequence in between the top and lower arrows is different between Case 14 and Case 198. The actual sequences of the cases are shown at the bottom of the figure.

M1080 that was recently reported by Maiden et al. (1991). This was the only non-P1.7,3 strain that we found among the 70 CSF samples analyzed thus far (data not shown).

**(f) Conclusions**

(1) We determined the nt sequence and the predicted structure of \textit{N. meningitidis} \textit{PorA} subtype P1.7,3 that is currently causing an epidemic in Chile. The sequence is consistent with the other published sequences (Barlow et al., 1989; Maiden et al., 1991; Van der Ley et al., 1991), but is unique in the variable region of loop 4, which we have shown to contain the subtype P1.3 epitope. Since the P1.7,3 subtype has been responsible for over 90% of the group-B disease in Northern Chile, this sequence information is critical for the development of the HNPCR strategy as well as the development and evaluation of potential vaccines for use in that area.

(2) We selected three sets of primers that are highly specific for \textit{N. meningitidis}. Of these, two sets of primers, p1 and p2, p3 and p4, specifically amplified a 1.1-kb and a 1-kb product of \textit{porA}, respectively, whereas the third set of primers, p5 and p6, specifically amplified the 0.5-kb size subtype P1.7,3-specific \textit{porA} product. Some strains of \textit{N. meningitidis} do not express any detectable PorA and are nontypable. Several of these were included in panels testing the primers. All were positive for \textit{porA} and yielded the predicted product with the exception of one isolate which was found to have a deletion of the gene (data not shown). \textit{N. gonorrhoeae}, which appears to harbor a silent \textit{porA} gene, however showed amplification of a 1.1-kb product with the outer primers p1 and p2, and a 0.6-kb product with inner nested primers p3 and p4, suggesting a potentially rearranged \textit{porA} sequence in this organism (data not shown). Barlow et al. (1989) previously suggested the presence of a \textit{porA}-related sequence on the basis of Northern blot analysis. This should not however affect the specificity of the assay or its usefulness in routine testing of clinical samples because the 0.6-kb product generated with p3 and p4 from \textit{N. gonorrhoeae} could be easily distinguished from the 1-kb size \textit{N. meningitidis} \textit{porA} product, and also \textit{N. gonorrhoeae} is unlikely to be present in the CSF samples. Determination of the subtype specificity of \textit{N. meningitidis} has been very useful from the point of view of vaccine efficacy evaluation. This is true because the vaccine is considered to be primarily a subtype-specific vaccine and may not protect against heterologous subtypes. The subtype-specific primers p5 and p6 could be simply replaced with another pair of subtype-specific primers in a different epidemiological setting, or if multiple subtypes are causing disease, a panel of subtype-specific primers could be used to determine the subtype of the isolates.

(3) The problems associated with amplification of DNA from low-copy-number samples have not been fully studied. From this study, it appears that minimizing the mispriming events, particularly those occurring during the initial cycles of PCR, played a critical role in amplifying a unique DNA fragment from a low-copy-number sample. We believe that mispriming is primarily due to two reasons. First, most protocols recommend the following sequence of steps for standard PCR: (i) boiling the complete PCR mixture, (ii) cooling the samples imme-

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**Panel A**

Case 14: TAACTT........TTACCCGACCTGCCTGGCC ........ACC
Case 198: TAACTTGTAGTAACCTTTACCTGCACCGTCGCCCGCTCGACC

**Panel B**

Case 14: AGCCCGAACCTGCAATATATATCTTTAGCACCATTGCAAGAATAC
Case 198: AGCCCGAACCTTTTTATAGCTACGCCCCATTTGGCCCGCAGA ...

Fig. 6. Determination of nt sequence of \textit{porA} amplified from CSF samples. The amplified \textit{porA} was directly sequenced using the PCR-directed sequencing (PDS) strategy described previously (Rao and Saunders, 1992). A 5 µl aliquot of the PCR mixture as in Fig. 5A was mixed with 5 µM each of four dNTPs, 30 pmol 32P-labeled primer, either 100 µM ddGTP, or 750 µM ddATP, or 750 µM ddTTP, or 1 mM ddCTP, 125 µM Mn2+ and 2.5 units of Taq polymerase. PCR cycling was done according to the conditions described earlier (Rao and Saunders, 1992). The labeled products were resolved by electrophoresis on a sequencing gel containing 6% polyacrylamide and 8 M urea. The gel was dried and autoradiographed by exposing to an X-OMAT AR (Kodak) film. Since the variable loops 1 and 4 are of most interest in determining the type specificity, sequencing was done using primers pl, p11 or p1II (Table 1) to cover these regions. (A) Sequence ladders were generated using loop-1 primer, pl. Note a 9-nt and a 3-nt insertion (bracketed with arrows) in Case 198 when compared to Case 14. (B) Sequence ladders were generated using loop-4 primer, p11. Note that the sequence in between the top and lower arrows is different between Case 14 and Case 198. The actual sequences of the cases are shown at the bottom of the figure.
diately on ice, and (iii) denaturation of DNA at 94°C to initiate the first PCR cycle (Arnheim and Erlich, 1992). Though these steps were intended to denature any protease or nuclease contamination in the crude sample, it is known to generate extensive mispriming (Mullis, 1991), which will be highly problematic while amplifying target DNA from a low-copy-number sample. This is because, during the cooling step and the subsequent denaturation step of the first PCR cycle when the temperature of the reaction mixture is rising from 4°C to 94°C, the DNA polymerase is active and would extend any template-primer complexes available in the reaction mixture. While the number of specific complexes is one or a few in a low-copy-number sample, there will be numerous nonspecific complexes that are formed as a result of short stretches of complementarity between the template and the 3'-end of the primer that is present in large excess. As a result, the nonspecific extension products predominate in the reaction mixture after the first PCR cycle (Fig. 3). We designed the HNPCR strategy to overcome this problem in two ways: first, the sample was denatured separately in the absence of any PCR components, and was then added to the PCR mixture; secondly, the first cycle of PCR was initiated by a hot-start procedure (D'Aquila et al., 1991) in which the DNA polymerase was added only after heating the samples to 90°C. The second problem in amplifying target DNA from a low-copy-number sample is the mispriming events that occur as a result of low stringency of annealing. This was minimized by designing the primers that have high estimated $T_m$ values, and having the $T_m$ values of the two primers close to each other. This allowed the performance of PCRs under highly stringent conditions with an annealing temperature that is close to the $T_m$ value. In some instances, we even used an annealing temperature that is the same as the estimated $T_m$ value without significantly affecting the efficiency of amplification (data not shown).

Even with the above modifications, we found that it was practically impossible to eliminate the mispriming events altogether. A DNA smear rather than a unique product was obtained after the first phase PCR in most of the experiments. This was because the synthesis of non-target DNAs from misprimed complexes is an inherent problem associated with the way the PCR technique works. In a low-copy-number sample, the ratio of DNA polymerase ($10^9$ molecules), or primers ($3 \times 10^{13}$ molecules each), to that of target DNA (1–10 molecules) is overwhelming high. Therefore, even under the most stringent PCR conditions, there will be a formation of transient unstable complexes between random regions of template and primers, and between primers. These complexes, at a certain frequency, will be converted to productive stable complexes by rapid addition of a few nt at the 3'-end of the primer by the highly active DNA polymerase that has a synthetic rate on the order of about 4000 nt per minute (Arnheim and Erlich, 1992). This problem will be further exacerbated if the test sample contained extraneous nonspecific DNA, which is almost always the case with a crude clinical sample. Indeed, it is known that *Taq* polymerase could prime DNA synthesis based on a two nucleotide homology, or in some instances no homology at all, generating primer dimers, multimers, and secondary recombination products in standard amplification reactions containing a high copy number of target DNA (Arnheim and Erlich, 1992). Reports that recommend extension of PCR cycling to 40–50 cycles to amplify DNA from a low-copy-number sample are counter-productive since this would lead to efficient amplification of nonspecific products that are predominantly present in the PCR mixture rather than the unique product (Mullis, 1991). We found that the only way to generate unique amplified product consistently from a low-copy-number sample is to do a nested PCR in addition to the modifications described above. Nested PCR selectively amplified the target DNA from a pool of target and non-target DNAs since the existence of complementary sequences on both ends of a non-target DNA is practically zero. Some mispriming events will undoubtedly occur in this step, but now since the starting copy number of target DNA is high, these events will be far outweighed by the specific amplification events.

(4) The power of this single copy DNA amplification strategy was further enhanced by extending it to generate nt sequence directly from the amplified DNA using a direct sequencing strategy. The composition of PCR mixtures was designed in such a way that the amplified DNA from the nested PCR could be directly used for sequencing without any time consuming template purification procedures. Therefore, using the complete strategy involving three successive phases of PCR, complete sequence of the target DNA was generated starting from one or a few DNA molecules. The sequence data generated from CSF samples of suspicious cases of the Iquique epidemic provided additional reliable data on the incidence of meningococcal meningitis among the volunteers enrolled in the study, and on the effectiveness of the vaccine.

(5) We believe that the HNPCR strategy could be applicable to a wide variety of genetic analyses in basic as well as clinical research labs. For example, it could be used for definitive diagnosis of genetic diseases at a very early stage of fetal development. It could also be used for definitive diagnosis of HIV in the initial stages of infection, or when the virus exists in a dormant stage. Because of minimal manual manipulations involved, the complete procedure could be automated for rapid analysis of a large number of samples.
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