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Typing *Neisseria meningitidis* by Analysis of Restriction Fragment Length Polymorphisms in the Gene Encoding the Class 1 Outer Membrane Protein: Application to Assessment of Epidemics throughout the Last 4 Decades in China

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A typing method was developed for *Neisseria meningitidis* serogroup A by analysis of restriction fragment length polymorphisms (RFLP) of the class 1 outer membrane protein gene (*porA*). By using appropriate primers, an approximately 1,116-bp fragment of the *porA* gene was amplified by PCR and then was digested with the restriction endonuclease *Msp*I. The digestion products were separated on 10% polyacrylamide gels and were stained with silver. One hundred three clinical isolates of group A *N. meningitidis* from 17 provinces of China collected over a 26-year period were analyzed. Results of *Msp*I-generated RFLP profiles of PCR-amplified *porA* genes were compared with those obtained by conventional serosubtyping. There was a band of about 400 bp common to all strains examined, and the 103 strains of serogroup A resulted in 22 unique RFLP patterns. The differences in bands could be observed mainly in the range of 120 to 280 bp. The smaller fragments were useful in distinguishing meningococci with the same serosubtype. Three epidemic periods were characterized by the presence of three distinct genotypes (a1, a2, and a3), accounting for 74.5% of the strains examined (3.88, 26.21, and 44.66%, respectively). Three predominant RFLP patterns were correlated epidemiologically with cycles of epidemic meningococcal meningitis and were well-matched to the predominant serosubtypes (P1.9, P1.7,10, and P1.9) that presented at the same prevalence cycles. The genotyping yielded information that allowed strains from one epidemic to be distinguished from those from another that would have been indistinguishable if only serotyping and serosubtyping were available. Therefore, the PCR-RFLP typing method was very useful in the epidemiologic investigation of group A meningococcal meningitis.

Serotyping and serosubtyping systems based on the recognition of epitopes on the major outer membrane proteins (MOMPs) with monoclonal antibodies (MAbs) have proven to be valuable tools for epidemiological investigations of *Neisseria meningitidis* (1, 29). The class 2 and 3 proteins define the serotypes and the antigenic variations of the class 1 protein define the serosubtypes of given isolates (9, 22). Several techniques in which MAbs were used for serotyping and serosubtyping of *N. meningitidis* have been reported (1, 5, 10, 32). These approaches require multiple assays, and some organisms are likely to be classified as nontypeable or nonsubtypeable because the complete range of antigenic variation is not covered by available MAbs. To improve discrimination among clinical isolates, other typing schemes have been developed by applying techniques of molecular biology to directly assess the genotypes of meningococci, e.g., multilocus enzyme electrophoresis (4, 20), fingerprints of chromosomal DNA (3, 6), and detection of restriction fragment length polymorphisms (RFLP) with cloned probes (8, 18).

PCR has proven to be an adequate means for the detection and typing of a variety of microorganisms (12, 27, 30, 31), and it has been used for the diagnosis of meningococcal disease (19, 24). RFLP analysis of PCR-amplified DNA has been shown to be of value in epidemiological typing of microorganisms (7, 25, 26). This technique, known as PCR-RFLP typing, allows quick and objective identification of the isolates, accommodates new variants, and does not require culturing of organisms. Recently, PCR-RFLP analysis of *porA* gene-encoding class 1 protein has been used for characterizing meningococci (17), and it provides a convenient and rapid alternative to standard subtyping methods currently used for *N. meningitidis*. In the present work, the *porA* gene was chosen for PCR-RFLP typing of 103 group A strains of *N. meningitidis*, and the results were compared with previous data resulting from serosubtyping (20).

MATERIALS AND METHODS

Meningococcal strains. One hundred three group A strains were examined. Polysaccharide vaccine strain 29201 was provided by L. Y. Wang (National Institute of Biological Products, Beijing, China), who received it from the Rockefeller University; B54 was from Mark Achtman (Max-Planck Institut fur Molekulare Genetik, Berlin, Germany). Seventy-seven patient strains and 24 carrier strains were collected from 17 provinces and municipalities of the People's Republic of China during 1966 to 1992.

DNA preparation. Meningococcal strains were cultured on heated blood agar at 37°C in 5% CO_2 for 18 h, and the cells from a plate were harvested into 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) to which 1 ml of 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM EDTA, and 1% Sarkosyl was added. Proteinase K was added to a final concentration of 100 μg/ml and incubated at 55°C for 2 h. A series of extractions were performed with equal volumes of phenol (saturated with TE buffer), phenol-chloroform (1:1, saturated with TE buffer), and chloroform. Boiled RNase A (Sigma) was added to a final concentration of 100 μg/ml, and the mixture was incubated for 2 h at room temperature. The mixture was extracted with phenol-chloroform and chloroform, and protein material was removed. After the addition of sodium acetate to a final concentration of 0.3 M, the mixture was overlaid with 1 volume of cold (−20°C)

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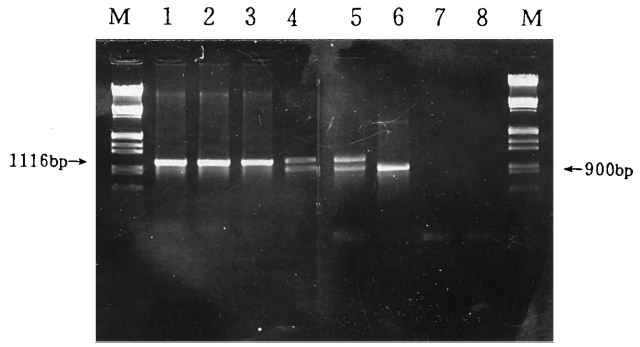

FIG. 1. Ethidium bromide-stained agarose gels containing PCR-amplified products of *porA* gene. Lanes: 1 to 6, *N. meningitidis* G275, 265, G311, 153, 79053, and 162; 7 and 8, *N. gonorrhoeae* F62 and 262; M, size markers (lambda DNA-*Eco*RI/*Hin*dIII).

isopropanol, and the DNA was washed once with 70% ethanol, dried, and resuspended in 1 ml of TE buffer. The DNA concentration and purity were estimated spectrophotometrically before use (28).

PCR amplification. The primers 21-(P67/93) 5′-CTG TAC GGC GAA ATC AAA GCC GGC GTG-3′ and 22-(P1182/1159) 5′-TTA GAA TTT GTG GCG CAA ACC GAC-3′, as Maiden et al. described (23), were synthesized by the Institute of Microbiology, Chinese Academy of Sciences. These primers were used to amplify a 1,116-bp fragment of the *porA* gene. The 50-μl reaction mixture contained 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.) and buffer supplied from the manufacturer, template DNA at 50 ng/μl, and 200 μM (each) dATP, dCTP, dGTP, and dTTP, and amplifications were carried out in a thermal cycler (LKB) for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by a further 4-min incubation at 72°C. PCR products were analyzed by electrophoresis of 5 μl of the amplification mixture on a 1% agarose gel and detected by staining with ethidium bromide.

Restriction endonuclease digestion and electrophoretic analysis. Ten-microliter amplified DNA samples were added to 1 μl of restriction endonuclease *Msp*I (Promega; 8 to 15 U/μl) and 1 μl of 10× reaction buffer according to the manufacturer's recommendations. The mixture was incubated at 37°C for 3 h, and then 2-μl digestion products were separated on 1.5-mm-thick 10% polyacrylamide gels (acrylamide-to-bisacrylamide ratio, 19:1) in Tris-borate buffer. Gels were silver stained (11); fixed for 45 min with agitation in 10% ethanol and 0.5% acetic acid; soaked for 2 h in 11 mM $AgNO_3$; rinsed in distilled water; reduced in 0.75 M NaOH, 0.1 M formaldehyde, and 2.3 mM sodium borohydride for 15 to 30 min; and stopped with 5% acetic acid when DNA bands were clearly visualized. Gels were photographed through blue and green filters.

RESULTS

PCR amplification of *porA* gene sequence. The chromosomal DNA samples from 103 group A strains of *N. meningitidis* were amplified by PCR with primers 21 and 22 encompassing the conserved regions of the *porA* gene. One hundred two strains produced a clearly visible band of 1,116 bp (Fig. 1, lanes 1 to 3) and 44 strains yielded an additional product of about 900 bp (lanes 4 and 5), whereas 1 strain (162) yielded only the 900-bp product (lane 6). Amplifications conducted on two strains of *N. gonorrhoeae* did not produce any product (lanes 7 and 8). The negative controls without added template DNA or other required reagents gave no products. To confirm the specificity of the amplification reaction, the amplified DNA was digested with restriction endonuclease *Eco*RI, and the 1,116-bp product was cleaved into 827- and 289-bp digestion fragments. The length and restriction sites of the 1,116-bp product were in concordance with the *porA* gene sequencing data (2). The product of 900 bp could not be cleaved with *Eco*RI. The amplification system was sensitive at the level of 1 pg of the chromosomal template DNA (data not shown).

Evaluation of restriction endonuclease digestion. On the basis of published sequences of the *porA* gene of the meningococcal strains (2, 21), 31 restriction endonuclease digestion product profiles were first simulated by computer analysis and the three (*Msp*I, *Taq*I, and *Hin*fI) that make multiple cuts in the sequence were tested against the amplified DNA samples. Of the three restriction endonucleases employed, *Msp*I was selected for RFLP typing because it gave the best discrimination and optimal numbers of bands. A random selection of 18 strains with different serosubtypes (P1.2, P1.7, P1.9, P1.10, and P1.7,10) were tested. *Msp*I digestion yielded 6 to 13 fragments and gave nine RFLP patterns which are able to distinguish all the tested subtypes, whereas *Taq*I and *Hin*fI yielded only 3 to 5 fragments and gave four and two RFLP patterns, respectively, and they could not distinguish the strains of P1.9 and P1.7,10, which were common subtypes in China. By *Msp*I digestion of the samples of 103 group A strains, the largest band of about 400 bp was a fragment common to all, whereas smaller fragments were more variable in the strains tested. To evaluate the intrastrain stability of the RFLP pattern, three randomly selected strains (153, 34, and 84001) from different epidemic years were subcultured for 40 generations and examined by the same procedures. The RFLP patterns of the *porA* gene remained identical to their originals (data not shown).

Differentiation of 103 group A strains. Examination and analysis of the *Msp*I restriction fragment profiles from 103 group A strains revealed 22 RFLP patterns (a1 to a22). Eighteen of these 22 distinct RFLP patterns are presented in Fig. 2. Applying these patterns, the typing results by RFLP analysis of

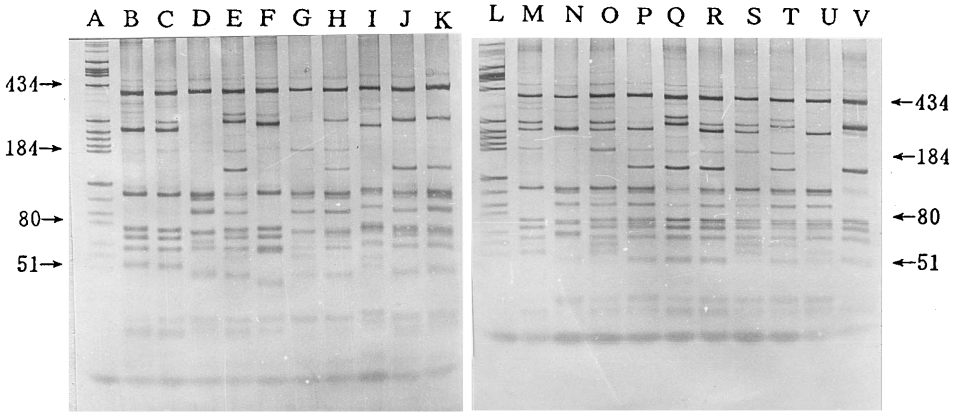

FIG. 2. *Msp*I digestion patterns of PCR-amplified *porA* gene from group A meningococci. Fragments were separated on a 10% polyacrylamide gel and stained with silver. Lanes: B, 84001; C, 87002; D, 162; E, 76024; F, 76002; G, 512; H, 79053; I, 84020; J, 519; K, 78059; M, 8821; N, 78044; O, 161; P, C307; Q, 274; R, 79096; S, 87053; T, G25; U, 153; V, 34; A and L, size markers (pBR322-*Hae*III).

TABLE 1. RFLP patterns of 103 strains of group A meningococci

Isolate	RFLP pattern	Clinical source	Yr of isolation	Origin	Sero-subtype
153	a1	Patient	1966	Henan	P1.9
154	a1	Patient	1966	Henan	P1.9
G62	a1	Patient	1967	Shanghai	P1.9
G70	a1	Patient	1967	Shanghai	P1.9
JS34	a2	Patient	1975	Jiangsu	P1.7,10
276	a2	Patient	1979	Jiangsu	P1.7,10
JS34	a2	Patient	1980	Jiangsu	P1.7,10
173	a2	Carrier	1977	Shanghai	NT[a]
G275	a2	Patient	1978	Shanghai	P1.7,10
320	a2	Patient	1979	Zhejiang	NT
3505	a2	Patient	1976	Tianjin	P1.7,10
3500	a2	Patient	1976	Tianjin	P1.7,10
78040	a2	Patient	1978	Sichuan	P1.7,10
78057	a2	Patient	1978	Hunan	P1.7,10
79202	a2	Patient	1979	Jiangxi	P1.10
80193	a2	Patient	1980	Jiangxi	P1.7,10
80186	a2	Patient	1980	Jiangxi	P1.7,10
29-64	a2	Patient	1980	Jiangxi	P1.7,10
80190	a2	Patient	1980	Jiangxi	P1.7,10
79094	a2	Patient	1979	Qinghai	P1.7,10
79099	a2	Patient	1979	Qinghai	P1.7,10
79101	a2	Patient	1979	Qinghai	P1.7,10
79095	a2	Patient	1979	Qinghai	P1.7,10
79097	a2	Patient	1979	Qinghai	P1.10
79120	a2	Patient	1979	Qinghai	P1.7,10
80014	a2	Patient	1974	Guizhou	NT
522	a2	Patient	1982	Guangxi	P1.7,10
520	a2	Patient	1982	Guangxi	P1.7,10
8522	a2	Patient	1985	Xinjiang	P1.7,10
SY3	a2	Patient	1982	Shenyang	P1.7,10
SY6	a2	Patient	1980	Shenyang	P1.7,10
SY40	a3	Patient	1982	Shenyang	P1.9
SY44	a3	Patient	1982	Shenyang	P1.9
SD3	a3	Patient	1984	Shandong	P1.9
SD9	a3	Patient	1984	Shandong	P1.9
265	a3	Patient	1984	Jiangsu	P1.9
266	a3	Patient	1984	Jiangsu	P1.9
84-11-001	a3	Patient	1984	Anhui	P1.9
84001	a3	Patient	1984	Sichuan	P1.9
84086	a3	Carrier	1984	Sichuan	P1.9
84004	a3	Patient	1984	Henan	P1.9
84031	a3	Patient	1984	Hebei	P1.9
85012	a3	Patient	1985	Hebei	P1.9
G311	a3	Patient	1985	Shanghai	P1.9
8508	a3	Patient	1985	Xinjiang	P1.9
85-5-6	a3	Patient	1986	Zhejiang	P1.9
87-3-1	a3	Patient	1987	Zhejiang	P1.9
87-3-3	a3	Carrier	1987	Zhejiang	P1.9
87-3-7	a3	Carrier	1987	Zhejiang	P1.9
87-6-1	a3	Patient	1987	Zhejiang	P1.9
87-4-1	a3	Patient	1987	Zhejiang	P1.9
87-7-6	a3	Patient	1987	Zhejiang	P1.9
87-7-1	a3	Patient	1987	Zhejiang	P1.9
87-13-1	a3	Patient	1987	Zhejiang	P1.9
87-13-2	a3	Carrier	1987	Zhejiang	P1.9
87-13-3	a3	Carrier	1987	Zhejiang	P1.9
87-22-1	a3	Patient	1987	Zhejiang	P1.9
87-26-1	a3	Patient	1987	Zhejiang	P1.9
87-21-1	a3	Patient	1987	Zhejiang	P1.9
85001	a3	Patient	1985	Henan	P1.9
150	a3	Carrier	1992	Henan	P1.9
138	a3	Carrier	1992	Henan	P1.9
139	a3	Carrier	1992	Henan	P1.9
112	a3	Carrier	1992	Henan	P1.9
LH1	a3	Patient	1992	Henan	P1.9
61	a3	Carrier	1992	Henan	P1.9
92001	a3	Patient	1992	Henan	P1.9
92002	a3	Patient	1992	Henan	P1.9
92010	a3	Carrier	1992	Henan	P1.9
92018	a3	Carrier	1992	Henan	P1.9
92024	a3	Carrier	1992	Henan	P1.9
92026	a3	Carrier	1992	Henan	P1.9
92036	a3	Carrier	1992	Henan	P1.9
92050	a3	Carrier	1992	Henan	P1.9
92051	a3	Carrier	1992	Henan	P1.9
92080	a3	Carrier	1992	Henan	P1.9
B54	a3	—[b]	—	Germany	P1.9
162	a4	Carrier	1966	Henan	NT
512	a5	Patient	1977	Guangxi	P1.7,10
79059	a5	Patient	1979	Hubei	P1.7,10
80060	a6	Carrier	1974	Guizhou	P1.7,10
76002	a7	Patient	1976	Henan	P1.7
84020	a8	Patient	1984	Henan	P1.9
78044	a9	Patient	1978	Sichuan	P1.2
79053	a10	Patient	1979	Hubei	P1.7,10
78059	a11	Patient	1978	Hunan	P1.7
519	a11	Patient	1982	Guangxi	P1.1
526	a11	Patient	1982	Guangxi	P1.15
G307	a12	Patient	1981	Shanghai	P1.10
29019	a13	Patient	1972	Shandong	P1.7,10
161	a14	Carrier	1966	Henan	P1.9
76024	a15	Patient	1976	Henan	P1.7,10
274	a15	Patient	1978	Henan	P1.7,10
79098	a15	Carrier	1974	Guizhou	P1.7,10
79096	a16	Patient	1979	Qinghai	P1.10
506	a17	Patient	1980	Guangxi	P1.7,10
G25	a17	Patient	1985	Guizhou	P1.10
87053	a18	Patient	1987	Anhui	P1.9
87002	a19	Patient	1987	Anhui	P1.9
8821	a19	Patient	1988	Zhejiang	P1.9
119	a20	Carrier	1986	Dandong	NT
88-6-1	a21	Patient	1988	Zhejiang	P1.9
29201	a22	—	—	United States	—

[a] NT, not subtypeable with MAb kit.
[b] —, unknown or not determined.

porA genes for 103 group A strains of *N. meningitidis* are shown in Table 1. Of the above patterns, a1, a2, and a3 were predominant (Fig. 2, lanes U, V, and B), accounting for 74.5% of the strains examined (3.88, 26.21, and 44.66%, respectively). They were common patterns both in the patient strains (57 of 77) and carrier strains (19 of 24). a1, a2, and a3 were the predominant patterns in the different cycles of nationwide epidemics of meningococcal meningitis in China during the 1960s, the 1970s, and 1980s, respectively, and each of them made up 66.67% (4 of 6), 58.62% (17 of 29), and 68.18% (45 of 66) of the Chinese strains in the different decades. The a1 pattern was found in Shanghai and Henan Provinces, a2 was in 12 provinces, and a3 was in 10 provinces.

The band profiles of three predominant RFLP patterns (a1, a2, and a3) are shown in Fig. 3. Sufficient variation in two higher-molecular-weight bands was observed to distinguish the a2 pattern (lanes E, F, and G) from a1 (B, C, and D) and a3 (H, I, and J). The a1 and a3 patterns exhibited a similar band profile, with an additional band of about 80 bp being observed in the a1 pattern. This band differentiated a1 from a3 genotypically, although they were phenotypically identical strains of the P1.9 serosubtype.

Examining the strains isolated under nonepidemic condi-

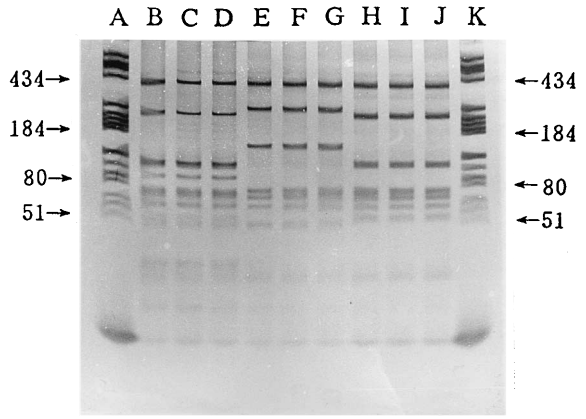

FIG. 3. The predominant RFLP patterns of group A *N. meningitidis* during the three prevalent cycles of epidemic cerebrospinal meningitis in China. Lanes show strains isolated in the 1960s (153 [B], G62 [C], and G70 [D], in the 1970s (78057 [E], G275 [F], and G276 [G]), and in the 1980s (G311 [H], 265 [I], and 266 [J]). Lanes A and K are size markers (pBR322-*Hae*III).

tions, we found various RFLP patterns that were divided into a4 to a22 RFLP patterns. Each pattern constituted a small proportion (0.97 to 2.91%) of the total strains tested. With the exception of the a11 and a15 patterns, which contained three strains each, the other patterns each contained only one to two strains. The B54 strain (clone III-1), which was a representative strain having wide international spread, gave an a3 RFLP pattern identical to that of the epidemic Chinese strains isolated after 1980. Strain 29201 from the Rockefeller University showed a pattern (a22) different from those of the Chinese strains.

Comparison of PCR-RFLP typing with MAb serosubtyping. Ninety-six of 103 group A strains constituted five serosubtypes, with seven strains proving nonsubtypeable (20). The predominant serosubtypes of the genotypic RFLP patterns a1, a2, and a3 were P1.9, P1.7,10, and P1.9, respectively, but some a2 strains were nontypeable and two strains were P1.10. Some strains with the same serosubtype may be divided into different RFLP patterns, e.g., 56 strains with the P1.9 subtype gave seven RFLP patterns and 31 strains with P1.7,10 gave eight RFLP patterns.

DISCUSSION

In order to develop useful epidemiological data to detect variations in serosubtypes, accurate and specific genotyping of *N. meningitidis* is required. For development of a protocol of typing *N. meningitidis* based on restriction endonuclease digestion of PCR products, the choice of a gene with demonstrated heterogeneity and discriminative restriction endonuclease sites is necessary. Expression of the class 1 protein of *N. meningitidis* is controlled by the *porA* gene. Some epitopes recognized by MAbs used for serosubtyping have been identified by the analysis of overlapping peptides (22). These epitopes are located in the variable regions (VR1 to VR3) of the protein sequence, with the majority of the peptide sequence variation found in VR1 and VR2. Analysis of sequence information combined with epitope mapping studies has revealed considerable epidemiological information not demonstrated by serosubtyping (23). Therefore, the *porA* gene was chosen as an epidemiological marker in this research for PCR-RFLP typing of *N. meningitidis*. In the first step of our procedure, amplified 1,116-bp products of the *porA* gene were obtained from 102 of 103 group A strains tested. Some strains yielded an additional 900-bp product that probably results from the amplification of a slightly smaller gene encoding the class 3 protein (21). If that is true, the results of our protocol may reveal some information associated with serotypes of these strains, but this remains to be studied.

The restriction site of *Msp*I is GGCC, which is multiply present in the *porA* gene, allowing for multiple cuts in the gene. Fortuitously, many *Msp*I restriction sites are in the variable regions and can be located in the published sequence (21). The sequence heterogeneity of these regions dictates alteration in epitopes for serosubtypes. Thus, RFLP patterns generally correlated with the serosubtypes of strains tested. Some strains with the same serosubtype were divided into different RFLP patterns, particularly a1 and a3, which were related to the epidemics of the 1960s and the 1980s in China, respectively. These strains belonged to the identical P1.9 serosubtype, but the a1 pattern had an additional band in the *Msp*I digestion profile. This observation reflects the power of molecular typing to discriminate among phenotypically similar strains. The differences between serosubtyping and PCR-RFLP typing may be explained as follows. Most meningococcal strains were complex subtypes which have two or more epitopes located at different domains of the class 1 protein. Some of the differences in the Chinese isolates had not been distinguished because of the lack of corresponding MAbs; for example, 56 strains with the P1.9 subtype gave rise to seven RFLP patterns. Secondly, *Msp*I makes multiple cuts outside the variable regions associated with serosubtypes, so the RFLP typing was able to reveal differences among strains with the same subtype in other sequence regions in the *porA* gene.

Meningococcal meningitis has been a serious problem in China. Every epidemic has basically spread from the north to the south of China. It appears in the form of a seasonal peak in March and April each year. Nationwide epidemics have occurred cyclically every 8 to 10 years, with peaks in 1959, 1967, 1977, and 1984 (13–16). Surveillance of serogroups of meningococci which caused the epidemics indicated that they were predominantly due to group A strains (95% of all cases), whereas group B or C occurred only in sporadic cases. The previous work showed that the cyclic epidemics were caused by the different predominant serosubtypes of group A strains (P1.9, P1.7,10, and P1.9) and the strains of predominant subtypes could periodically spread (14, 20). The results of this work showed that the predominant RFLP patterns, a1, a2, and a3, were related to the three prevalent cycles of the epidemics in the 1960s, 1970s, and 1980s, respectively, and matched well with the predominant serosubtypes. Most strains in the epidemics of the 1960s and 1980s were subtype P1.9, but they were divided into distinct a1 and a3 patterns by PCR-RFLP typing. After 1980, a3 was the predominant RFLP pattern of epidemic group A strains in China, accounting for 67.16% (45 of 67). It probably was derived from a1. The point mutation probably occurred in the *porA* gene to generate the amino acid change in the other undetermined epitope, resulting in "immunoescape" or a virulence change, facilitating the spread of a3 strains and increasing the incidence of meningococcal disease in 17 provinces in 1984. The group A polysaccharide vaccine was used in China on a large scale for children under 15 years old to control the epidemics after 1984, and the incidence has decreased gradually in the past few years.

The results of this work demonstrate that PCR-RFLP analysis of the *porA* gene is a highly reproducible and discriminatory technique for typing *N. meningitidis*. It reveals that each of the nationwide epidemics in China was caused by a group A strain with a different predominant RFLP pattern. It suggests

that cyclical epidemics are probably due to strains with mutations in the specific region of the *porA* gene that produce changes of antigenicity and/or pathogenicity for the group A *N. meningitidis*. PCR-RFLP analysis of the *porA* gene can provide another marker system which will be useful to epidemiological investigations of epidemic cerebrospinal meningitis.

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