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Increase in meningococcal disease associated with the emergence of a novel ST-11 variant of serogroup C *Neisseria meningitidis* in Victoria, Australia, 1999–2000

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SUMMARY

In the years 1999–2000, there was an increase in the incidence of meningococcal disease in Victoria, largely caused by *Neisseria meningitidis* serogroup C. This change was associated with a shift in age distribution of cases, with relatively more disease appearing in the 15–29 year age group, and with 40/58 serogroup C isolates in 2000 exhibiting a new macrorestriction pattern (pattern A). Thirty-four of 52 pattern A isolates tested displayed the novel phenotype C:2a:P1.4, and were consistently porA VR type P1.7-2,4 by DNA sequencing. Nine of 10 representative pattern A isolates analysed displayed a housekeeping gene allele profile (ST-11) that is characteristic of the electrophoretic type (ET)-15 variant that has caused outbreaks in Canada, the Czech Republic and Greece. Meningococci belonging to the ST-11 complex that were isolated in Victoria prior to 1999 did not display either restriction pattern A or PorA VR type P1.7-2,4.

INTRODUCTION

Neisseria meningitidis is a cause of bacterial meningitis and septicaemia, often resulting in death and disability [1]. Invasive meningococcal disease (IMD) may occur as endemic sporadic cases, linked cases, or as epidemics. The unpredictability and severity of IMD causes great public anxiety, and in all states of Australia is a notifiable disease.

Epidemics and hyperendemic waves of IMD are usually associated with the spread of particular virulent clones [2]. Worldwide, in recent years, there have been several instances of a rise in serogroup C disease associated with a lineage of hypervirulent meningococci known as electrophoretic type 15 (ET-15), a variant belonging to the ET-37 clonal complex, or lineage. This lineage was identified by the technique of multilocus enzyme electrophoresis (MLEE) [3]. Strains of this complex typically have the antigenic formula (serogroup:serotype:serosubtype) C:2a:P1.5,2. The ET-15 variant strain first emerged in 1986 in Canada. Subsequently, it appeared in the Czech Republic in 1993, causing a major outbreak [4, 5]. More recently, outbreaks of serogroup C disease attributable to ET-15 have been reported in Greece and in Australia [6, 7].

In recent years the tracking of the evolution and spread of hyperinvasive lineages of meningococci has been facilitated by multilocus sequence typing (MLST) and porin gene sequencing (*porA* and *porB* VR typing). MLST, a nucleotide sequence based method, has emerged as an alternative to MLEE. Multilocus methods are particularly valuable in view of high frequency transformation events occurring in meningococci [8]. With MLST, invasive lineages of meningococci can be identified by their profile of housekeeping gene alleles known as the sequence type

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(ST). Isolates belonging to the ET-37 clonal complex most commonly exhibit the ST-11 profile by MLST analysis [9]. The ET-15 variant is distinguished by a rare allele at the fumarase locus, that can be detected by DNA sequencing, but is outside the standard MLST *fumC* locus target region [10]. In the past, the serotype and serosubtype have remained fairly homogeneous within the ET-37 complex, but genotype diversification has been observed to occur during the spread of disease [11]. Macrorestriction analysis using pulsed-field gel electrophoresis (PFGE) enables the tracking of short-term recombination and diversification of genotype [12].

In the years 1999–2000 in Victoria, Australia, there was a substantial increase in reported cases of IMD accompanied by a change in the ratio of serogroup B to serogroup C cases. We undertook a serological and molecular analysis of this change in pattern of disease. We show here that the increase in cases of IMD is largely the result of the appearance of a new recombinant variant of *Neisseria meningitidis* that belongs to the ST-11 clonal complex, which carries the ET-15 fumarase marker and displays the antigenic profile C:2a:P1.4.

MATERIALS AND METHODS

Bacterial cultures

Isolates of *N. meningitidis* from normally sterile sites (cerebrospinal fluid, blood and joint fluid) were submitted routinely from laboratories throughout the state of Victoria. For the period 1994 to 2000, 685 cases of IMD were notified to the Department of Human Services (DHS) and in 461 of these cases, isolates were submitted to the state public health laboratory. Meningococcal isolates were stored at −70 °C in 1·6% tryptone (Difco) containing 20% glycerol. They were revived by sub-culture onto saponin-lysed blood agar plates (Difco GC base) supplemented with Vitox (Oxoid) and incubated overnight in 5% CO_2.

Serological analysis

Serogrouping was performed by agglutination using capsular polysaccharide-specific polyclonal antibodies prepared locally in rabbits, by a method obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Serotyping and serosubtyping were conducted using a dot blot technique with a panel of monoclonal antibodies provided by Rijksintituut voor Volksgezondheid en Milieuhygiene (RIVM, National Institute of Public Health and Environmental Protection), Bilthoven, The Netherlands [13]. The panel of antibodies used recognised serotype-specific epitopes: 1, 2a, 2b, 4, 14 and 15 on the PorB porin, and the serosubtype specific epitopes: P1.1, P1.2, P1.4, P1.5, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P1.14, P1.15 and P1.16 on the PorA porin.

Pulsed-field gel electrophoresis (PFGE)

Genomic fingerprints were generated using *Bgl*II restriction endonuclease [14]. Separations of *Bgl*II restriction fragments of whole *N. meningitidis* genome were performed in 0·5 × TBE buffer on a 1·05% agarose gel using the CHEF-DR III apparatus (Bio-Rad, USA). Switching times were 5–10 s at 6·0 V cm^{-1}, with a 120° included angle and a run time of 22 h. The gels were then photographed and macrorestriction patterns were visually aligned, given sequential classification by letter and then sub-grouped by appending a number for minor pattern differences. The degree of genetic relatedness was then judged using the protocol set out by Tenover *et al.* [15].

DNA amplification and nucleotide sequence determination

All nucleotide sequences were determined by direct cycle sequencing of polymerase chain reaction (PCR) product. Boiled cell suspensions were used as template for generating specific PCR product. PCR fragments were purified by polyethylene glycol precipitation, and then sequenced using BigDye Terminator Sequence chemistry (Applied Biosystems), and separated using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). Forward and reverse sequences were completed and trace files were analysed and aligned using *Sequencher* software (GeneCode Corp. © 1998).

Multilocus sequence typing

Amplification and sequencing primers used were as described by Maiden *et al.* for the six gene loci *aroE*, *pdhC*, *abcZ*, *pgm*, *gdh* and *adK* [9]. For the seventh locus [16], *fumC*, the following primers were used for both amplification and sequencing: *fumC*-S3 (5′-TCG GCA CGG GTT TGA ACA GC-3′) and *fumC*-S4 (5′-

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CAA CGG CGG TTT CGC GCA AC-3′). For each isolate an allelic profile and sequence type was determined by entering data at the MLST website (http://www.mlst.net/), developed at the Wellcome Centre for the Epidemiology of Infectious Diseases, Oxford University, UK.

The *fumC* locus was further characterized by sequencing a separate DNA fragment to determine the sequence at position 640. G to A base substitution at position 640 distinguishes ET-15 meningococci from other ET-37 complex strains [10]. The amplification primers used were *fumC*-A1 (5′-CAC CGC ACA CGA CAC GAT GG-3′) and *fumC*-A2 (5′-ACG ACC AGT TCG TCA AAC TC-3′). The PCR product was sequenced using the primer *fumC*-P3 (5′-GCT AAA AGC CCT GCG CGA C-3′).

PorA and PorB variable region (VR) typing

Direct *porA* sequence determination of variable regions VR1 and VR2 permitted us to predict the amino acid sequence of epitopes on variable immunogenic regions of the PorA protein. This provided us with a high-resolution alternative to serosubtyping. The nomenclature used here for reporting *porA* variants was developed by the collaborative efforts of the Wellcome Trust Centre for the Epidemiology of Infectious Disease at the University of Oxford and researchers working on *N. meningitidis* classification worldwide [17].

A PCR primer pair, designed from *porA* nucleotide sequence (Accession no. AE002492) was used to amplify a 668 bp fragment of *porA* gene that contained VR1 and VR2. The primers were named *porA*p1 (5′-TTA CCG CCC TCG TAT TGT CCG CA-3′) and *porA*p2 (5′-GTA AAT CAG ACC GGC ATA ATA CAC-3′). The same primers were used for sequencing amplified *porA* product. The PorA VR amino acid sequences were determined and each VR type was assigned a number to describe the family, followed by a hyphen and a second number to describe the variant of each family type. The PorA VR type is defined as a particular combination of the VR1 and VR2, such as P1.5-1,10-4. PorA VR type designations are available at the Wellcome Trust Centre for the Epidemiology of Infectious Diseases, Oxford University (http://outbreak.ceid.ox.ac.uk/PorA-VR/vr_index.htm).

The *porB* gene encoding the serotype antigen of *N. meningitidis* was sequenced over a region containing variable regions VR1, VR2, VR3 and VR4. PorB VR type designations were assigned to each of the four regions according to the scheme devised by Sacchi *et al.*, for example C,Eb,2a,C [18]. Amplification and sequencing primers were chosen from homologous regions of *porB* nucleotide sequence (Accession numbers U07193 and X67940). The amplification primers were *porB*p1 (5′-ATG AAA AAA TCC CTG ATT GCC CTG-3′) and *porB*p2 (5′-TTA GAA TTT GTG ACG CAG ACC GAC-3′). Sequencing primers were *porB*p1, *porB*p2, and additionally *porB*p4 (5′-CGT ATT GTA CGC TGC CGC TGA GG-3′) and *porB*p5 (5′-GCC TCA GCG GCA GCG TAC AAT AC-3′).

Epidemiological data analysis

Cases of IMD were identified through the Victorian Notifiable Infectious Diseases Surveillance (NIDS) system. Demographic data for all notified cases were provided by public health staff at the state Health Department. Descriptive analysis of these data was conducted in Epi Info, Version 6, software [19]. Proportions were compared using the Yates χ^2 test corrected for continuity.

RESULTS

Serogroup epidemiology

Figure 1 shows that a substantial increase in the incidence of IMD occurred in Victoria in 1999. In this year there were 137 notifications, the highest number reported to DHS since 1956. There were 162 notifications in 2000. Incidence (notified cases) per 10^5 population increased from 1·29 in 1998 to 3·44 in 2000. Both the total number of notified cases (which included cases diagnosed on clinical evidence alone), and numbers of culture confirmed cases (typically 80% of notified cases) increased to a similar degree.

In the few years prior to 1999, invasive disease was caused predominantly by serogroup B strains (see Fig. 1), but in both 1999 and 2000, significantly more cases were caused by serogroup C ($P < 0{\cdot}01$) which increased from 17% of isolates in 1998, to 46% in 1999 and 55% in 2000. Cases caused by serogroup B strains in 1999–2000 occurred in numbers that were similar to those of the preceding period.

PFGE analysis and serology

All of the serogroup C culture isolates obtained in the period 1996–2000 were analysed for genetic similarity

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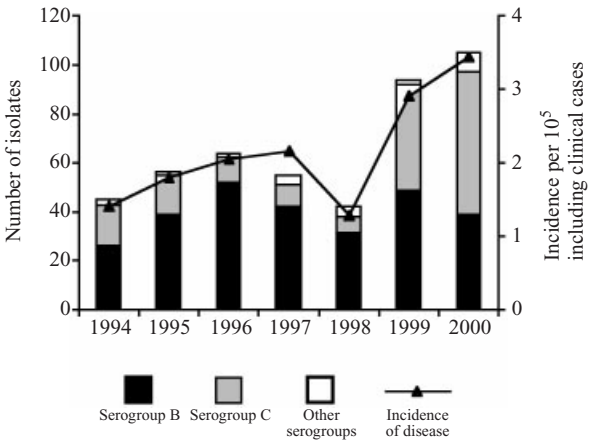

Fig. 1. Numbers of cultured meningococcal isolates and incidence of notified cases of IMD in Victoria, 1994–2000. Incidence per 105 values include cases diagnosed on clinical criteria alone, in addition to those confirmed by microbial culture.

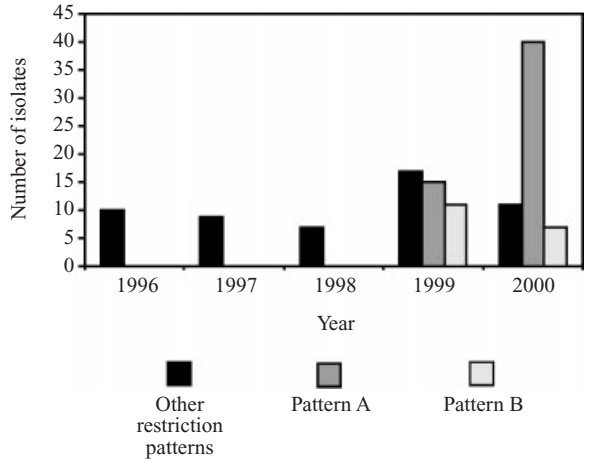

Fig. 2. Comparison of the frequency of PFGE pattern A and B serogroup C isolates with all other serogroup C isolates, 1996–2000. The category 'Other restriction patterns' includes PFGE patterns C–F and any remaining patterns exhibited by serogroup C isolates.

by genomic DNA macrorestriction using *Bgl*II enzyme, and fragment patterns were assessed by PFGE. Six major macrorestriction PFGE patterns were detected. These have been referred to as patterns A–F. PFGE patterns C–F were found from the period 1996–2000, together with a variety of unrelated macrorestriction types (data not included). Patterns A and B were two new patterns that appeared for the first time in 1999.

In Figure 2, numbers of isolates with PFGE patterns A and B are compared with numbers of all other serogroup C isolates from the period 1996–2000. PFGE pattern A isolates were by far the most common category in the period 1999–2000 and made a significant contribution to the increase in serogroup C disease ($P < 0{\cdot}01$). In 2000, 40 of 58 (69%) serogroup C isolates exhibited the PFGE pattern A.

Pattern A isolates typically exhibited a C:2a:P1.4 antigenic profile, previously undetected in Victoria. Of 52 Pattern A isolates fully typed by serology, 34 were C:2a:P1.4, 16 were C:2a:nst, 1 was C:2a:P1.2 and 1 was C:2a:P1.5,2. In contrast, pattern B–E isolates were typically C:2a:P1.5(P1.2) or C:2a:nst. Pattern F isolates were C:2b:P1.2. The failure to obtain specific serosubtypes (nst) for all strains may reflect in part the lack of sensitivity of the dot blot method used for typing. Over time, variations of 2–3 bands appeared in PFGE pattern A amongst isolates, indicating that genetic diversification was occurring in the outbreak strain. These variant patterns are referred to as A1, A2, A3 and A4 (see Table 1).

DNA sequence analysis

MLST housekeeping gene profiles were determined on representative isolates from each of the six serogroup C macrorestriction types in order to identify their genetic lineage (see Table 1). Five of these macrorestriction types (patterns A–E) yielded the ST-11 allelic profile (*abcZ* 2, *adK* 3, *aroE* 4, *fumC* 3, *gdh* 8, *pdhC* 4, *pgm* 6), whereas pattern F displayed the unrelated allelic profile ST-66 (*abcZ* 2, *adK* 3, *aroE* 7, *fumC* 2, *gdh* 34, *pdhC* 5, *pgm* 2). Repeated analysis (see Table 1) of different serogroup C isolates of the same PFGE pattern consistently yielded the same MLST sequence type, confirming that macrorestriction analysis as used here was a reliable measure of genetic relatedness of these isolates. Of the 10 pattern A isolates examined for their MLST profile, 9 were ST-11, and the tenth exhibited the closely related housekeeping gene profile ST-475, which shares 6 of 7 housekeeping alleles with ST-11.

Analysis of the *fumC* allele at nucleotide position 640 is relevant for assessing relatedness of ST-11 isolates to ET-15 lineage strains [9]. Table 1 shows that pattern A–D isolates all carry the ET-15 marker $fumC_{640}$ A substitution, whereas pattern E isolates carry the ET-37 version of this marker.

The same set of representative serogroup C isolates (see Table 1) were also examined by DNA sequencing of their *porA* and *porB* genes to determine the PorA VR type and the PorB VR type. The *porA* sequence covered the two variable regions (VR1 and VR2)

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Table 1. *Molecular analysis of representative isolates of major serogroup C PFGE patterns from 1996–2000.*

		Allele profiles detected by DNA sequencing			
PFGE pattern	No. of isolates	MLST sequence type	*fumC*$_{640}$ substitution	PorA VR type	PorB VR type
A1	4	11	A	7-2,4	C, Eb, 2a, C
A2	2	11	A	7-2,4	C, Eb, 2a, C
A3	2	11	A	7-2,4	C, Eb, 2a, C
A4	1	11	A	5,2*	C, Eb, 2a, C
	1	475	A	7-2,4	C, Eb, 2a, C
B	2	11	A	5,2	C, Eb, 2a, C
C	2	11	A	5-1,10-4	C, Eb, 2a, C
D	2	11	A	5,2	C, Eb, 2a, C
E	2	11	G	5,2	C, Eb, 2a, C
F	1	66	n.d.	5,2	C, Ea, 2b, C

* PorA gene sequence analysis was performed on 35 pattern A isolates; 33 were PorA VR type P1.7-2,4 and 2 were P1.5,2 (both PFGE pattern A4).
n.d. = not determined.

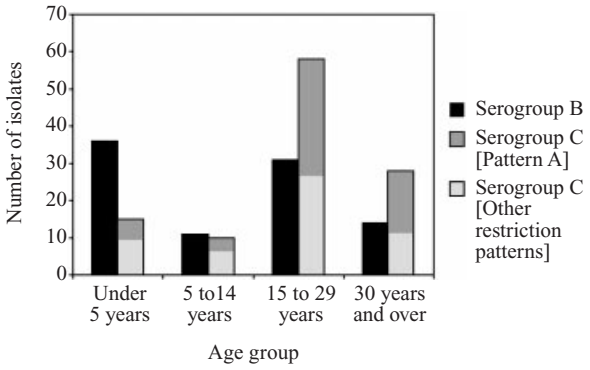

Fig. 3. Cases of serogroup B and C meningococcal disease in Victoria by age group, 1999–2000. Serogroup C (other restriction patterns) includes all serogroup C isolates except those giving macrorestriction pattern A.

coding for the two epitopes that determine serosubtype PorA. VR typing by this DNA sequencing method provided typing data even when isolates were serologically non-subtypable, and yielded results that were concordant with the serological analysis, given that in PorA VR type P1.7-2,4 the VR1 epitope is serologically hidden [20]. The data showed that the pattern A isolates generally encode a PorA VR type previously reported to occur in isolates belonging to the ST-41 complex (Lineage 3) that is distinct from ST-11 [21]. As far as we know this P1.7-2,4 PorA VR type has not previously been associated with any infections caused by ST-11 meningococci.

PorA DNA sequence analysis was carried out on 35 pattern A isolates; 33/35 were shown to encode the PorA VR type P1.7-2,4. The remaining two pattern A isolates encoded PorA VR type P1.5,2. This suggests that either the ancestral PorA VR type of the pattern A isolates was P1.5,2 or that the P1.7-2,4 had been replaced with P1.5,2 by recombination. ST-41 complex isolates with phenotypic profile B:4:P1.4 and PorA VR type P1.7-2,4 were common in Victoria during the period 1996–2000 (details not presented). More extensive sequence analysis covering the entire *porA* coding region together with some upstream sequencing was carried out on two representative pattern A ST-11 isolates and two representative ST-41 isolates. All four organisms shared an identical *porA* DNA region of over 1360 nucleotides.

The *porB* gene which codes for the serotype antigen was sequenced over four variable regions in each of the representative isolates (Table 1). No PorB VR type variation was found amongst the ST-11 isolates, that were all serologically serotype 2a. The PorB VR type of the unrelated ST-66 isolate was consistent with it being serotype 2b.

Demographic characteristics of cases

Figure 3 shows the age related incidence of IMD for serogroup B and serogroup C disease in Victoria. In the older age group (15–29 years), serogroup C was significantly more often the cause of IMD than serogroup B ($P = 0{\cdot}04$). In contrast, serogroup B disease was more common in the under 5 year age group. Disease caused by PFGE pattern A isolates occurred more frequently in the older age group. In

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1999, there were more cases of IMD in teenagers and young adults than in infants 1–4 years old. The ratio of notified cases aged 5 years and over to those under 5 years in the period 1999–2000 was 2·73 (219/80), whereas in the three preceding years 1996–1998, the ratio averaged 1·48 (151/102) [22].

Although not statistically significant, the case fatality rate for serogroup C cases during the period 1999–2000 was higher (12·2%) than that of serogroup B cases (8·0%), ($P = 0{\cdot}43$). Although there were significant differences between males and females in the pattern of serogroup B and C case distribution within years, overall there was not a significant difference between males and females.

DISCUSSION

The rise in incidence of IMD in Victoria to 3·44 per 10^5 in the year 2000 is comparable in severity to changes occurring during the Canadian ET-15 outbreak [5], and that in the Czech Republic [4]. In Canada the annual incidence rose steadily from 0·8 to 2·0 per 10^5 between 1985 and 1992, and in the Czech Republic it was 1·3, 1·9 and 2·2 per 10^5 in 1993, 1994 and 1995 respectively. In both these outbreaks young adults experienced significantly greater increases in disease than children aged under 5 years. In the Czech Republic the ratio of cases aged 5 years and over to those aged under 5 years rose from 0·8 in 1992 to 2·3 in 1994. A comparable situation has been reported recently in Greece where a steady increase in incidence per 10^5 from 0·57 in 1993 to 2·65 in 1997 was due to the virulent ET-15 variant with antigenic profile C:2a:P1.2(P1.5) first noted in Canada and the Czech Republic. In Greece in 1993–4, 74% of IMD cases were in children aged 5 years and under, but in 1995–7 only 45% were in this younger age group [7].

We believe that we are seeing a similar situation in Victoria with the emergence in 1999 of a variant serogroup C meningococcus belonging to the ST-11 clonal complex. The Victorian situation is different in that the new variant encodes a PorA VR type P1.7-2,4. Both a change in the clonal structure of isolates and a pronounced shift in the age distribution of cases towards the 15–29 year age group were noted in Victoria, as was the case in Canada, the Czech Republic and Greece. Other studies have shown that these two characteristics typically distinguish epidemic disease from sporadic disease [23].

Several different observations are consistent with the postulate that a novel serogroup C variant of the ST-11 complex emerged in Victoria in 1999, and that this postulated new recombinant strain has inherited a DNA fragment covering the *porA* region encoding PorA VR type P1.7-2,4 which is identical to that found frequently in serogroup B, ST-41 complex strains.

Firstly, in support of this postulate, a distinctive PFGE fingerprint (pattern A) that was not detected prior to 1999 was repeatedly present in serogroup C isolates in 1999–2000 (Fig. 2). Secondly, in conjunction with the detection of this new pattern, serogroup C isolates showed a high degree of clonality in 1999–2000. Thirdly, repeated DNA sequence analysis of these new serogroup C pattern A isolates showed consistently that they were identical at all nine genetic loci analysed (see Table 1). These identical loci comprised the seven housekeeping gene loci characteristic of allelic profile ST-11 plus the *porB* and *porA* loci. DNA sequencing of a larger set of PFGE pattern A isolates revealed that 33/35 carried the same *porA* DNA encoding PorA VR type P1.7-2,4. None of the ST-11 isolates of PFGE patterns other than pattern A encoded the P1.7-2,4 PorA VR type or displayed P1.4 phenotype. All these observations confirm our hypothesis that a new variant of *N. meningitidis* related to ET-15 but displaying the PorA VR type P1.7-2,4 had become a major cause of increased IMD in Victoria.

ET-15 strains have been shown previously to undergo genomic modifications during the spread of disease, leading to heterogeneity in genotype and phenotype [24]. Capsule switching from serogroup C to B has been demonstrated among ET-15 isolates in Canada and the Czech Republic, and some non-subtypable ET-15 isolates have been seen, but none has been reported to date with serosubtype P1.4. [25, 26]. We noted the DNA sequence encoding PorA VR type P1.7-2,4 in isolates of the ST-41 clonal complex shared an identical DNA sequence over 1360 bp with that found in our variant ST-11 isolates, implying that the *porA* regions in these two different lineages shared a recent common ancestor. Meningococci belonging to the ST-41 clonal complex (Lineage 3) had been frequently isolated from cases of IMD in Victoria in the 1990s, and in other countries in 1980–2000 [27, 21].

Previous studies on isolates of the ST-41 complex bearing the P1.7-2,4 porin variant have shown that the VR1 epitope (previously referred to as P1.7h or P1.7b) is hidden from antibodies [20, 21]. There is also evidence from vaccine studies [28] that the VR2 loop

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of P1.7-2,4 is poorly immunogenic. Poor antibody responses against *porA* determinants may thus play a role in the rise of meningococcal disease caused by organisms carrying the PorA VR type P1.7-2,4. Reduced herd immunity in the 15–24 year age group has been cited as a factor in the emergence of ET-15 related disease in a community [26].

As yet we have not been able to investigate systematically other relevant antigens in our isolates or the patterns of meningococcal strain carriage in our population. Other causative factors of IMD outbreaks, which include host susceptibility, environmental and socio-economic conditions, and infectious cofactors also require further investigation [5, 29].

Newer DNA sequencing based methods such as MLST and porin gene sequencing have proved powerful when used in combination with traditional strain characterization methods. In this study, MLST methodology allowed us to relate our observations of PFGE patterns to an international database of lineages, and had practical advantages for timely identification of hypervirulent, hyperinvasive clones [16, 30]. The completeness of porin epitope characterization in terms of predicted amino acid sequences of VR loops allowed us to document the complete conservation of PorA and PorB epitopes in the ST-11 variant isolates, which would not have been revealed by serology alone.

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