Epidemiol. Infect. (2004), **132**, 637–645. © 2004 Cambridge University Press
DOI: 10.1017/S0950268804002316 Printed in the United Kingdom

Re-emergence of meningococcal disease in Taiwan: circulation of domestic clones of *Neisseria meningitidis* in the 2001 outbreak

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(*Accepted 17 February 2004*)

SUMMARY

The annual incidence of meningococcal disease (meningitis and septicaemia) in Taiwan was $0{\cdot}94/10^5$ population in 1953. It then declined to below 0·001 from 1980 to 1987, and re-emerged in 2000 with a rate of $0{\cdot}07/10^5$ population. In 2001 there was a further increase in incidence (43 cases, $0{\cdot}19/10^5$). Of 43 isolates of *Neisseria meningitidis* available for this study, including 41 from patients treated in 2001, three (7·0%) were penicillin insensitive (MIC $\geqslant 0{\cdot}12\,\mu$g/ml), though all were β-lactamase negative; 16 (37·2%) were resistant to trimethoprim–sulphamethoxazole (MIC $\geqslant 4/76\,\mu$g/ml). Serogrouping and genotype analysis revealed nine domestic clones. None of the 43 patients had any relationship (travel or contact history) with the 2000 or 2001 Hajj pilgrimage. Epidemiological information and typing results suggested wide dissemination of a limited number of domestic clones of *N. meningitidis*, manifesting as serogroups W-135, B and Y. Two clones of serogroup W-135 involved in the outbreak were genetically distinct from the 2000 or 2001 Hajj-related W-135 clone.

INTRODUCTION

Neisseria meningitidis, a Gram-negative diplococcus, causes a spectrum of clinical features ranging from nasopharyngeal colonization and respiratory tract infections to meningitis, fulminant meningococcaemia, and multi-organ failure [1–5]. Meningococcal disease incidence varies worldwide [1, 2, 4]. In the United States since the 1960s, the annual incidence has ranged from 0·9 to 1·5 cases/10^5 population [1]. In 1997, approximately 500 000 cases of meningococcal disease were reported worldwide [1]. A large outbreak occurred in the African 'Meningitis Belt' in 1996 with more than 150 000 cases and a case fatality rate of approximately 10% [6]. Although there are at least 13 known serogroups, most reported sporadic cases or outbreaks of meningococcal disease are caused by serogroups A, B, or C [1, 3, 7–11]. In recent years, some less common serogroups, such as Y and W-135, have been associated with outbreaks in the United States and elsewhere [1, 12–15]. International spread of serogroups A and W-135 in association with the Hajj pilgrimage has been noted [11, 16–20].

Penicillin has long been recommended as the antimicrobial of choice for treating meningococcal disease while rifampicin and sulphonamides have been used extensively for prophylaxis of contacts and carriers [1, 21, 22]. *N. meningitidis* isolates exhibiting

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decreased sensitivity or resistance to these drugs have been reported from many countries, although the prevalence of resistance varies widely [21, 23–25].

In Taiwan, active surveillance of a range of communicable diseases including meningococcal disease (laboratory-confirmed meningitis or bacteraemia) has been conducted by the Center for Disease Control (CDC, formerly the National Institute of Preventive Medicine) of Taiwan since the 1950s. From 1996 to 2000, between 10 and 20 cases of meningococcal disease were reported annually. Previous studies from different parts of Taiwan showed a low incidence of *N. meningitidis* septicaemia and meningitis in both children and adults [26–29]. In studies of military recruits in 1974–1975, the meningococcal nasopharyngeal carriage rate was reported to be 13·7% [30].

An unusual increase in the number of cases of meningococcal disease was reported to the Taiwan CDC during the period 1 January 2001 to 31 December 2001. We investigated the incidence and epidemiology of meningococcal disease in Taiwan from the 1960s up to and including 2001. As part of the study we characterized the isolates responsible for the 2001 outbreak, determining their serogroup distribution, susceptibility to antimicrobial agents and molecular relatedness.

METHODS

Patients and bacterial isolates

Patients with a diagnosis of invasive meningococcal disease (bacteraemia and/or meningitis) reported to Taiwan CDC during the period from 1 January 1950 to 31 December 2001 were included in the study. Relevant demographic and clinical data, as well as records of the serogroup distribution of the isolates were available for 128 patients treated during the period from 1 January 1995 to 31 December 2001. Nasopharyngeal swabs were cultured for *N. meningitidis* from all persons who had close contact with the patients. Rifampicin (600 mg every 12 h for 2 days) was given to all exposed individuals.

In all, 43 isolates recovered from 43 patients with meningococcal disease (meningitis and/or bacteraemia) were preserved for further study. Of these isolates, 41 were from 41 patients seen during the period from 1 January 2001 to 31 December 2001; the remaining two isolates were from patients seen in March 1998 and March 2000 respectively. Isolates were identified by conventional biochemical methods.

Table 1. *Results of* in vitro *antimicrobial susceptibility testing for 43 isolates of* Neisseria meningitidis *recovered from patients in Taiwan* (*1 January 1998 to 31 December 2001*)

	MIC (μg/ml)		
Drug	Range	50%	90%
Penicillin	⩽0·03–0·5	⩽0·03	0·06
Ampicillin	0·06–4·0	0·25	1·0
Ceftriaxone	⩽0·03	⩽0·03	⩽0·03
Cefepime	⩽0·03–0·06	⩽0·03	⩽0·03
Imipenem	⩽0·03–0·25	⩽0·03	0·03
Meropenem	⩽0·03–0·06	⩽0·03	0·03
Faropenem	⩽0·03–0·12	⩽0·03	0·06
Ciprofloxacin	⩽0·03	⩽0·03	⩽0·03
Moxifloxacin	⩽0·03–0·06	⩽0·03	⩽0·03
Gatifloxacin	⩽0·03–0·06	⩽0·03	⩽0·03
Rifampicin	⩽0·03–0·06	⩽0·03	0·06
Erythromycin	⩽0·03–1·0	0·12	0·25
Clarithromycin	⩽0·03–0·5	0·06	0·25
Chloramphenicol	0·06–1·0	0·5	1·0
Tigecycline	⩽0·03–0·12	0·06	0·12
Trimethoprim–sulphamethoxazole*	0·12–8	2·0	4·0

* Data shown are concentrations of trimethoprim.

Serogroups

Serogrouping by the agglutination test (Murex Biotech Ltd, Dartford, UK) was performed at the Taiwan CDC with standard grouping sera for capsular types A, B, C, X, Y, Z and W-135.

Anti-microbial susceptibility and determination of β-lactamase production and *penA* gene polymorphism

The 15 antimicrobial agents shown in Table 1 were provided by their manufacturers for use in this study. Minimum inhibitory concentrations (MICs) of these antimicrobial agents were determined using the agar dilution method according to NCCLS guidelines [31]. The isolates were grown overnight on trypticase soy agar plates supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD, USA) at 37 °C. When testing trimethoprim–sulphamethoxazole, 5% lysed horse blood was used. Bacterial inocula were prepared by suspending freshly grown bacteria in sterile normal saline and adjusted to a 0·5 McFarland standard. For susceptibility testing, Mueller–Hinton agar supplemented with 5% sheep blood (BBL Microbiology Systems) was used. Using a Steers replicator (Mast Group Ltd, Bootle, UK), an

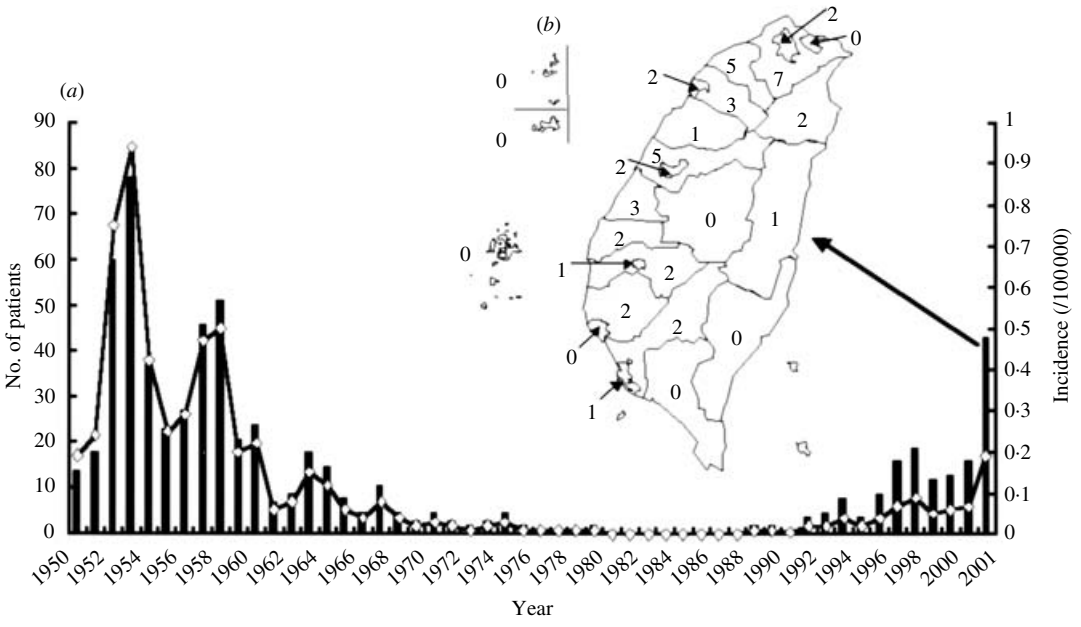

Fig. 1. (*a*) Annual number of cases and incidence (per 10^5 of population) of invasive meningococcal disease in Taiwan from 1961 to 2001 and (*b*) distribution of cases of invasive meningococcal diseases in different parts of Taiwan in 2001. ■, No. of patients; —◇—, incidence.

organism density of 10^4 c.f.u./spot was inoculated onto the appropriate plate with various concentrations of antimicrobial agents and incubated at 35 °C in 5% CO_2 atmosphere for 24 h. *Streptococcus pneumoniae* ATCC 49619 was included as the control strains.

β-lactamase production was sought using the Cefinase disk test (BBL Microbiology Systems). The polymorphism of the *penA* gene was analysed by investigating restriction endonuclease patterns for amplified *penA* following digestion with three enzymes (*Taq*I, *Hpa*II, and *Hae*III) [32].

Strain typing

Genotypes were identified by random amplified polymorphic DNA (RAPD) patterns generated by arbitrarily primed PCR (APPCR) and pulsed-field gel electrophoresis (PFGE) as previously described [33–35]. The four random primers used in the APPCR analysis were M13 [5′-GAGGGTGGCGGTTCT-3′ (Gibco–BRL, Gaithersburg, MD, USA)], ERIC1 [5′-GTGAATCCCCAGGAGCTTACAT-3′ (Gibco–BRL)], OPH-03 (5′-AGACGTCCAC-3′), and OPH-09 (5′-CTGACCAGCC-3′) (Operon Technologies Inc., Alameda, CA, USA). The restriction enzymes used for PFGE analysis were *Bgl*II, *Spe*I, and *Nhe*I [34–36].

To interpret RAPD patterns, both faint and intensive bands were included. RAPD patterns were considered identical only if they differed by no more than one band. Interpretation of PFGE profiles (pulsotypes) was in accordance with the criteria previously described [37].

Definitions

Isolates were defined as being of the same clone (highly related isolates) if they had identical serogroups, RAPD patterns, and pulsotypes.

RESULTS

Trend of invasive meningococcal disease in Taiwan

A total of 659 cases of meningococcal disease (meningitis and bacteraemia) were reported to the Taiwan CDC during the study period (1950–2001 inclusive). The annual incidence peaked in 1953 (78 cases, 0·94/10^5 population), reaching a high level again in 1959 (51 patients, 0·52/10^5 population), then decreasing in the following 30 years (Fig. 1*a*). The disease nearly disappeared from 1975 to 1987 (only seven cases reported during 1975–1979 and no cases during 1980–1987), re-emerging in 1988 and increasing

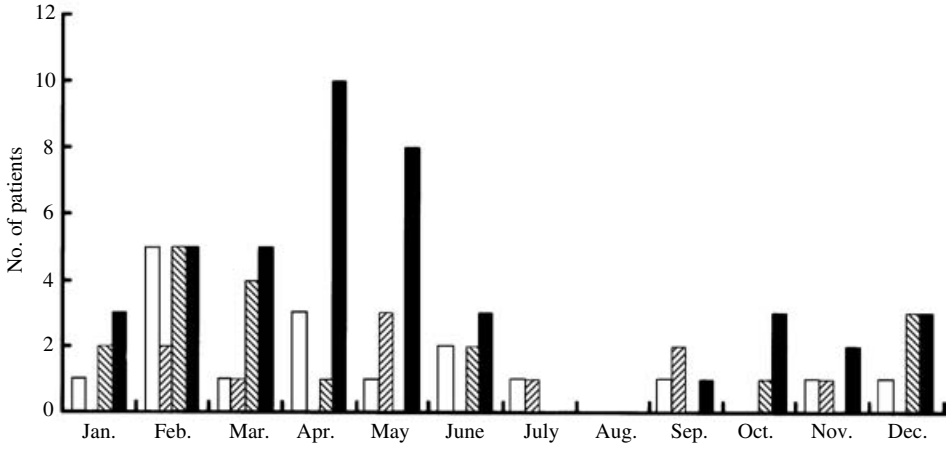

Fig. 2. Monthly numbers of patients with invasive meningococcal disease in Taiwan from 1 January 1998 to 31 December 2001 (84 patients in total). □, 1998; ▨, 1999; ▧, 2000; ■, 2001.

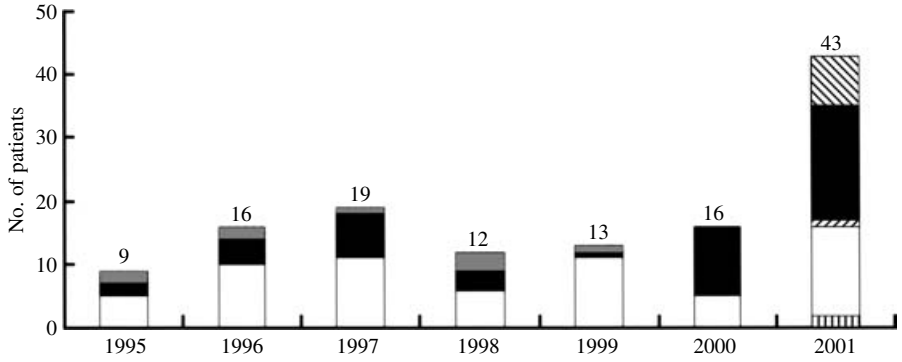

Fig. 3. Serogroup distribution among 128 isolates of *N. meningitidis* recovered from 128 patients in each year from 1995 to 2001. Serogroups of isolates from 1995 to 2000, which were not preserved for this study, were obtained from Center for Disease Control, Department of Health, Taiwan. ▥, A; □, B, ▨, C; ■, W-135; ▧, Y; ▩, non-typable.

in 1997 (19 patients, $0{\cdot}09/10^5$ population). From 1 January 2001 to 31 December 2001, a substantial increase in the number of cases of meningococcal disease (43 cases, $0{\cdot}19/10^5$) was noted. These 43 cases were dispersed in different parts of Taiwan (Fig. 1*b*).

Among the 128 patients with a diagnosis of meningococcal disease from January 1995 to December 2001, the mean age was 19·4 years; 43 (33·6%) were aged from 11 to 30 years, 40 (31·3%) were aged below 1 year, and 67 (52·3%) were male. The disease occurred all year round (except for August) and cases were concentrated from December to June (Fig. 2). Among the 43 patients treated in 2001, the mean age was 19·4 years, 21 (48·9%) were male, nine (20·9%) were military recruits, and most (72·1%) developed the disease during the period from February to June. The majority of the 128 isolates belonged to serogroups B (48·4%) and W-135 (35·9%). In 1999, serogroup B isolates accounted for 11 out of 13 (84·6%) of all isolates but in 2001 this prevalence decreased considerably (32·6%), while the prevalence of serogroup W-135 (41·9%) increased and serogroup Y emerged (18·6%) (Fig. 3).

The mortality was highest in 1999 (30·6%), whereas no patient died in 1997 or 1998. The overall mortality of the 43 patients seen in 2001 was 25·6%: 42·8% of patients infected with serogroup B isolates and 37·5% of patients with serogroup W-135 isolates died. None of these 43 patients was in close contact with another case. None of the 2001 patients had travelled internationally and none had contact with travellers to Saudi Arabia during 2001 or attended the 2000 or 2001 Hajj or had any contact with the pilgrims returning from the 2000 or 2001 Hajj.

Table 2. *Microbiological characteristics of 43 isolates of* Neisseria meningitidis *recovered from 1998* (*one isolate*), *2000* (*one isolate*), *and 2001* (*41 isolates*)

		Genotype (no. of isolates)			
Serogroup	No. of isolates tested	RAPD pattern (M13/ERIC1/ OPH-03/OPH-09)	Pulsotype (*Spe*I/*Bgl*II/*Nhe*I)	Year of isolation	Clone
W-135	18	W1 (17)	w1 (17)	2001	1
		W2 (1)	w2 (1)	2001	2
Y	8	Y1 (8)	y1 (8)	2001	3
B	15	B1 (9)	b1 (9)	2001	4
		B2 (4)	b2 (6)	2001	5
		B3 (1)	b3 (1)	1998	6
		B4 (1)	b4 (1)	2000	7
A	1	A1 (1)	a1 (1)	2001	8
C	1	C1 (1)	c1 (1)	2001	9

Antimicrobial susceptibilities and β-lactamase production

The MICs of 15 antimicrobial agents for the 43 isolates of *N. meningitidis* are shown in Table 1. When the MIC break-points for susceptibility and resistance used for *S. pneumoniae* or *N. gonorrhoeae* were applied to *N. meningitidis* [31], all strains tested were susceptible to all agents tested, except for penicillin and trimethoprim–sulphamethoxazole. Sixteen isolates (37·2%) were resistant to trimethoprim–sulphamethoxazole (MICs, $\geqslant 4/76\,\mu$g/ml). Three isolates (3·2%) were relatively insensitive to penicillin (all had MICs of $0{\cdot}5\,\mu$g/ml) and these isolates were all β-lactamase negative.

Pulsotypes and RAPD patterns

Nine clones were identified amongst the 43 isolates of *N. meningitidis*, based on the PFGE profiles (pulsotypes) and RAPD patterns (Table 2 and Fig. 4). Four major clones, i.e. clones 1 (serogroup W-135, 17 isolates), 3 (serogroup Y, 8 isolates), 4 (serogroup B, 9 isolates), and 5 (serogroup B, 4 isolates) were found at different times and in different regions of Taiwan in 2001 (Fig. 5). All three penicillin-insensitive isolates belonged to clone 1. Two isolates recovered in 1998 and 2000 were genetically different from isolates found in 2001 and belonged to clones 6 and 7 respectively. None of the pulsotypes of the nine clones were identical or closely related to those reported from other countries, including that of the epidemic Hajj-related W-135 clone (MenW135) or (W)ET-37 clone [16, 34, 35].

penA gene polymorphism

The three penicillin-insensitive isolates (clone 1) had identical restriction profiles of *penA* that were different from those of the three penicillin susceptible isolates. The DNA sequences of the *penA* amplicon from the three penicillin-resistant strains and three randomly selected susceptible isolates with penicillin MICs of $\leqslant 0{\cdot}03\,\mu$g/ml (clone 1), $\leqslant 0{\cdot}03\,\mu$g/ml (clone 2), and $0{\cdot}06\,\mu$g/ml (clone 4), were subsequently determined. All three *penA* sequences from the susceptible strains showed more than 98% similarity. However, the *penA* from the resistant strains showed much lower similarity ($\sim$75%) to those of other strains.

DISCUSSION

The reasons for the re-emergence of meningococcal disease in Taiwan in recent years and the upsurge in 2001 are unclear. Spread of some meningococcal clones nationwide seems to be the most likely explanation for the substantial increase in the number of cases over a short time. However, increased alertness and better handling of clinical samples (particularly cerebrospinal fluids) and better recognition of *N. meningitidis* by microbiology laboratories (due to programmes of intensive training for clinical microbiology staff and frequent proficiency tests monitored by the Department of Health of Taiwan during the period), may also have contributed to increased recognition. More efficient and timely notification of cases to the CDC by clinicians and microbiology staff

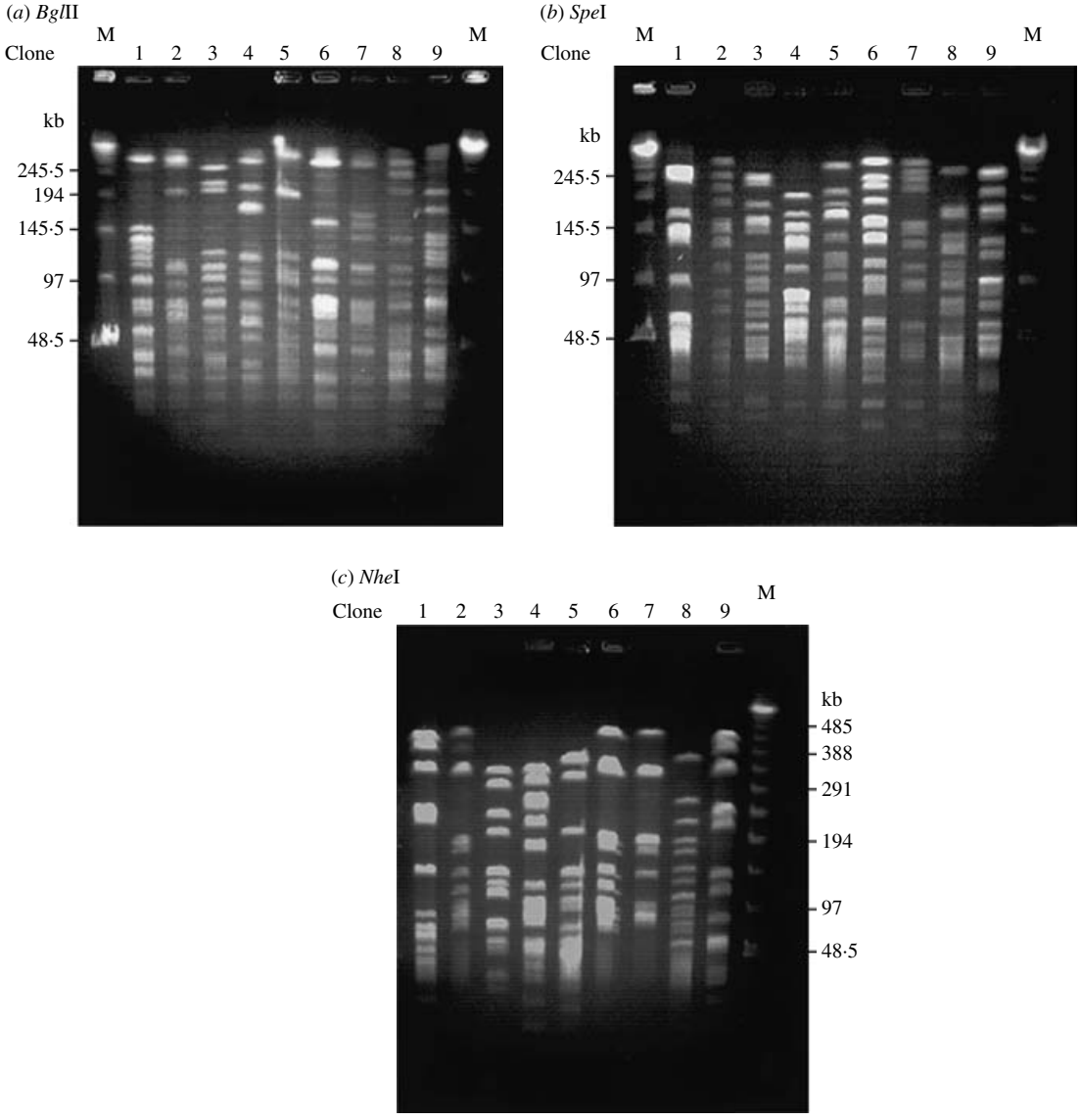

Fig. 4. Representative pulsed-field gel electrophoresis profiles digested with *Bgl*II (*a*), *Spe*I (*b*), and *Nhe*I (*c*) of nine clones identified in 43 *N. meningitidis* isolates collected from March 1998 (one isolate), March 2000 (one isolate), and 1 January 2001 to 31 December 2001 (41 isolates) in Taiwan. Lane M, molecular size markers; lanes 2–10, pulsotypes of clones 1–9 respectively.

may also have contributed to the apparently increased incidence.

In addition to the re-emerging nature of meningococcal disease in Taiwan, three important points were demonstrated in this study. First, in addition to the predominance of serogroup B, the emergence of serogroup Y isolates in 2001 and the increase of serogroup W-135 in 2000–2001 were noteworthy. Secondly, epidemiological information and typing results indicated that wide dissemination of a limited number of domestic (Taiwan) clones of *N. meningitidis*, particularly serogroups W-135 and Y, contributed to the Taiwan 2001 outbreak [16, 34–36]. Although the two isolates recovered before 2001 were genetically distinct from the clones isolated in 2001, the lack of more isolates from years prior to 2001 makes it impossible to know if any of the 2001 clones were prevalent in earlier years. The two clones of serogroup W-135 involved in this outbreak were genetically unrelated to the W-135 clone associated with the epidemic in the 2000 or 2001 Hajj pilgrimage [16, 34–36].

Finally, the low prevalence of penicillin insensitivity (3·2%) and high prevalence of trimethoprim–sulphamethoxazole resistance (37·2%) among recent isolates is interesting, particularly as it occurred in a

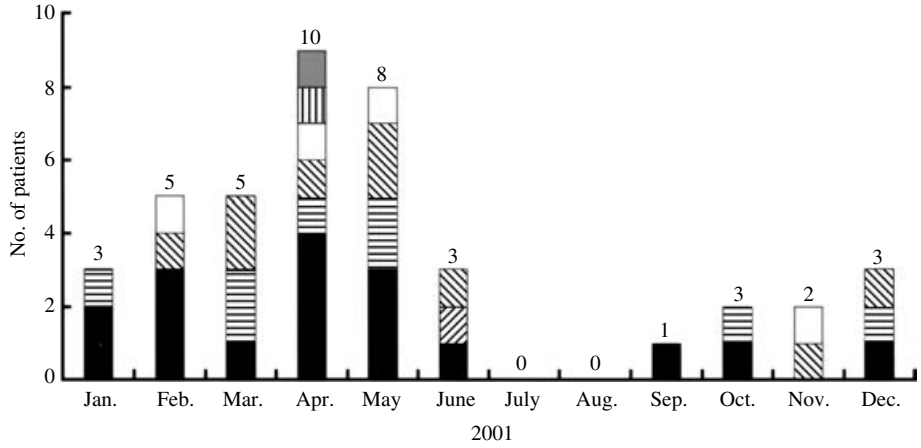

Fig. 5. Distribution of seven meningococcal clones by month in 2001 [plus one each in 1998 (March, b3) and 2000 (March, b4)]. PFGE analysis was not performed on two isolates: one serogroup A isolate (April 2001) and one serogroup B isolate (October 2001). ■, 1 (W-135); ▨, 2 (W-135); ▤, 3 (Y); ▧, 4 (B); □, 5 (B); ▥, 8 (A); ■, 9 (C).

region with a high prevalence of penicillin and/or trimethoprim–sulphamethoxazole resistance among strains of *N. gonorrhoeae* and *Streptococcus pneumoniae* [38, 39]. In this study, the three penicillin-insensitive isolates were β-lactamase-negative and exhibited alteration of the *penA* sequence. Previously reported extremely rare penicillin-resistant isolates of *N. meningitidis* were β-lactamase producers [1, 21, 39].

MIC break-points of susceptibility and resistance to antimicrobial agents for *N. meningitidis* isolates have not been recommended by the NCCLS [31], and the criteria used in this study and most other studies followed the NCCLS criteria for *S. pneumoniae* and *N. gonorrhoeae* [31]. The reported prevalence of penicillin insensitivity varies widely from country to country, ranging from $<10\%$ in most countries (including Taiwan), to 46% in Spain [1, 21]. Most reports of strains insensitive to penicillin had MICs ranging from 0·1 to 1·28 μg/ml (most due to altered penicillin-binding protein-2, PBP-2) although isolates exhibiting MICs of 4–256 μg/ml (most due to β-lactamase production mediated by chromosome or plasmids) have been reported [21, 23, 24].

Roberts and Knapp [40] demonstrated that conjugative transfer of β-lactamase plasmids occurred from *N. gonorrhoeae* to *N. meningitidis* and commensal *Neisseria* species. Other mechanisms such as decreased expression of class 3 porin and decreased affinity of PBP-1 may contribute to the resistance of *N. meningitidis* to penicillin [41]. Further study is needed to determine whether other mechanisms may have contributed to penicillin insensitivity in our isolates, although the penicillin MICs of these isolates were lower than those of previously reported resistant isolates associated with β-lactamase production and multiple resistance mechanisms [21, 23, 24].

In comparison with other isolates of clone 1, the penicillin-insensitive isolates had identical pulsotypes, RAPD patterns and serogroups but had different penicillin MICs and *penA* sequences. These findings suggest the possibility of horizontal transfer of *penA* genes between these penicillin-resistant isolates and other bacteria [41].

Resistance to sulphonamides, rifampicin, ciprofloxacin and ceftriaxone is of concern in the management of patients with meningococcal disease [1, 21, 42]. Resistance to sulphonamides (sulphadiazine or trimethoprim–sulphamethoxazole) has been reported worldwide with rates of up to 30% in the United Kingdom and 54% in the United States [21, 43, 44]. A previous report from Taiwan on nasopharyngeal carriage isolates in military recruits also showed a high level of resistance (54·1%) to sulphadiazine [30]. Our isolates were all susceptible to the latter three agents. Other agents such as carbapenems, macrolides, newer fluoroquinolones, and chloramphenicol were also active against our isolates. These findings support observations made in previous studies [21, 23–25, 42]. The MIC_{90} of our isolates for tigecycline, a newer semi-synthetic glycylcyline, was 0·12 μg/ml, consistent with the findings of Gales and Jones [45]. Penicillin is still recommended as the drug of choice for treating meningococcal disease in Taiwan.

Several typing methods have been applied to *N. meningitidis* to evaluate strain relatedness in isolates collected during outbreaks [16, 33, 35, 46]. Multilocus enzyme electrophoresis is considered the gold standard for typing this organism and multilocus sequence typing and PFGE are also used as alternatives [32, 35, 46]. However, these methods are labour-intensive and time-consuming. Recent data suggest that RAPD analysis generated by APPCR can provide rapid and reliable discrimination for typing isolates of *N. meningitidis* [33]. In our study, RAPD typing using four primers for the 43 isolates of *N. meningitdis* had similar discriminatory power to typing by PFGE.

Close contacts are at increased risk of contracting meningococcal disease and warrant chemoprophylaxis [47, 48]. Meningococcal vaccination is beneficial for individuals at high risk or travellers to countries recognized as having epidemic disease caused by a vaccine-preventable serogroup [47, 48]. During the 2001 outbreak in Taiwan three persons, who had close contact (frequently slept or had meals in the same dwelling) with one of the 41 patients, had nasopharyngeal colonization with *N. meningitidis*. The three isolates from the three contacts and the patient's isolate all belonged to serogroup B, indicating the possibility of close relatedness of the four isolates, although the three isolates were not preserved for further typing.

The persistently high annual proportion of serogroup B isolates causing invasive meningococcal disease in the last 6 years and the predominance of serogroup B (54·3%) among nasopharyngeal colonizers in a study of military recruits in 2001 (data shown elsewhere) suggest that the quadrivalent vaccine (A, C, Y and W-135) has a limited role in controlling carriage by homologous serogroups. If safe and effective serogroup B meningococcal vaccines were available, evaluation of their use in Taiwan would be warranted.

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