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Emergence of Reduced Chlorhexidine Susceptibility in
Methicillin-Resistant *Staphylococcus aureus* at a Teaching Hospital

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Abstract

We studied the chlorhexidine susceptibility, molecular typing, and the distribution of the *qacA/B* gene of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained in 1990, 1994, 1995, and 2002 at the National Taiwan University Hospital (NTUH). The prevalence rate of MRSA with a high minimum inhibitory concentration (MIC) of chlorhexidine ($\geq 4 \mu\text{g/mL}$) was found to increase rapidly during this 13-year period, from 3.3% in 1990 to 66.7% in 2002. Nearly all of the MRSA isolates with high chlorhexidine MICs carried the *qacA/B* gene and belonged to a single molecular type. The prevalence rate of MRSA isolates with high chlorhexidine MIC correlates well with that of MRSA isolates belonging to the molecular type. We conclude that the prevalence rate of MRSA with high chlorhexidine MIC increased rapidly during past 13 years at the NTUH, which was associated with an endemic strain, carrying the *qacA/B* gene, spreading in the hospital.

Key words: methicillin-resistant *Staphylococcus aureus*, MRSA, chlorhexidine, pulsed-field gel electrophoresis, *qacA/B*

Introduction

The first clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) at the National Taiwan University Hospital (NTUH) was found in 1981 (1). Thereafter, the nosocomial MRSA infection rate at NTUH increased rapidly, especially during 1990s and the prevalence rate of methicillin resistance in *S. aureus* isolates causing nosocomial infection at the NTUH remained at 60 – 80% during the past 10 years (2-3). The possible reasons for this high prevalence rate of MRSA nosocomial infection included poor adherence of healthcare workers (HCWs) to isolation precautions leading to transfer of MRSA between patients via their colonized hands or the inanimate environment (4, 5), use and/or abuse of broad-spectrum antibiotics (3,6,7), increase of therapeutic modalities that breach the integrity of the mucocutaneous system, and introduction of an endemic MRSA strain into the institute (5, 8-10).

To prevent the transfer of MRSA between patients via HCWs' hands, antiseptics such as chlorhexidine were used for hand washing (11). Based on the history of antibiotic resistance, some authors predicted confidently and then proved the emergence and wide spread of resistance to antiseptics in *S. aureus*, especially MRSA (12-16).

Resistance of *S. aureus* to antiseptics, including chlorhexidine, is conferred by two gene families, *qacA/B* and *smr* (14). The *qacA/B* gene confers a high-level resistance to antiseptics, and the *smr* gene family confers a low-level resistance. With the existence of chlorhexidine resistance in *S. aureus*, hand disinfectants based solely on chlorhexidine (such as Hibiscrub) might become limitedly effective against *S. aureus*, which probably would not stop the spread of MRSA in a medical institute using this kind of hand disinfectant for hand washing of health care workers (HCWs) (17).

Hibiscrub (Zeneca Ltd. Co., Macclesfield Cheshire England, UK), which contains chlorhexidine gluconate (4% w/v) as its major antibacterial component, has been an important disinfectant for hand washing for more than 20 years at the NTUH. The present longitudinal study was carried out to understand the changes of susceptibility to chlorhexidine and the prevalence of MRSA isolates carrying the *qacA/B* gene at a teaching hospital with a high prevalence rate of MRSA nosocomial infection and long-term chlorhexidine use.

Methods

Hospital settings

NTUH is a major teaching hospital with a 2,200-bed capacity located in northern Taiwan. It offers both primary and tertiary medical cares.

Bacterial strains

A total of 240 MRSA isolates, with 60 isolates in each year, were randomly selected from the MRSA isolates obtained from clinical specimens, including blood, sputum, pus, discharge, and other body fluids in 1990, 1994, 1995, and 2002 at the NTUH. No duplicate isolates from a single patient or an outbreak were included.

Bacterial identification

Identification of *S. aureus* was based on colony morphology on trypticase soy agar supplemented with 5% sheep blood (BBL, Microbiology Systems, Cockeysville, MD, USA), Gram stain, and a positive Staphylase test (Oxoid Ltd, Basingstoke, England). The *S. aureus* isolates obtained were screened for methicillin resistance using the standard disk diffusion method, with use of Mueller-Hinton agar (BBL, Microbiology Systems) and 1 µg oxacillin disk after incubation for 24 hours at 35°C (18).

Susceptibility test

Chlorhexidine minimum inhibitory concentration (MIC) was determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (19). The chlorhexidine MIC for MRSA isolates carrying the *qacA/B* gene is typically equal to or greater than 4 µg/mL according to previous reports (15, 20, 21). Therefore, an isolate with a high chlorhexidine MIC was defined in this study as having a MIC of chlorhexidine equal to or greater than 4 µg/mL.

Pulsed-field gel electrophoresis (PFGE)

Molecular typing of all 240 MRSA isolates was done using PFGE according to the methodology described in details previously (22). The banding pattern of the PFGE results was interpreted according to the criteria suggested by Bannerman et al and Jorgensen et al (23, 24). Various types and subtypes of the PFGE pattern were named as those we used in our previous studies (22).

Detection of the *qacA/B* gene

Bacterial DNA was extracted using the InstaGene™ matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Briefly, an isolated bacterial colony was suspended in 1 mL of autoclaved water in a microfuge tube and then centrifuged for 1 minute at 6,000 g. The supernatant was removed. The pellet was then added into 200 µL of InstaGene™ matrix (Bio-Rad) and vortexed, followed by heating at 56°C for 15 minutes. The sample was vortexed again and heated at 100°C for 8 minutes and then centrifuged to pellet the matrix. The

supernatant was used in subsequent polymerase chain reactions (PCR). The following primers were used for the amplification of the *qacA/B* genes: the forward primer 5'-CTATGGCAATAGGAGATATGGTGT, and the reverse primer 5'-CCACTACAGATTCTTCAGCTACATG (14). PCR was performed as described elsewhere (14). DNA sequences of the PCR products were determined by Applied Biosystem 377 DNA sequencer (PE-ABD, Foster City, CA).

Results

Chlorhexidine susceptibility

The chlorhexidine MICs of the 240 MRSA isolates collected from different years are shown in Table 1. The MIC₉₀ increased from 2 µg/mL in 1990 to 8 µg/mL in 2002. The prevalence rate of MRSA isolates with high chlorhexidine MIC (≥ 4 µg/mL) increased markedly during the study period, from 3.3% to 66.7%.

PFGE patterns

The PFGE banding patterns of the 60 MRSA isolates collected in 1990 revealed great diversity, with no predominant pattern evident. In contrast, analysis of the banding pattern of the 60 MRSA isolates isolated in 1994 revealed the predominance of the type B strain. This strain comprised 31 of the 60 isolates (51.7%), with type A and type C (consist of only one subtype, C1) comprising 17(28.3%) isolates and seven (11.7%) isolates, respectively. The six remaining isolates belonged to six other minor types (types D, E, F, G, H, and I).

Among the 60 isolates isolated in 1995, 32 isolates (53.3%) belonged to three subtypes (C1, C2, and C3) of type C, 15 isolates (25%) belonged to type B, and the other 13 isolates (21.7%) belonged to four other minor types (types A, D, F, and J).

The PFGE banding pattern of the 60 MRSA isolates obtained in 2002 revealed that the type C remained the predominant PFGE type, comprising 21 isolates (35%) (belonging to two subtypes, C1, and C2). Thirteen isolates (21.6%) belonged to type K, six isolates (13.3%) belonged to two subtypes (L1 and L2) of type L, nine isolates (15%) belonged to two subtypes (M1 and M2) of type M, and the other nine isolates belonged to six other minor types (types N, O, P, Q, R, and S). This result was similar to our previous study (22). The PFGE banding patterns of type A, B, C1, C2, C3, K, L1, L2, M1, and M2 are shown in Figure 1.

Distribution of the *qacA/B* gene

The *qacA/B* gene was detected in 0, 7 (11.7%), 32 (53.3%), and 44 (73.3%) isolates of the MRSA isolates obtained in 1990, 1994, 1995, and 2002, respectively. Nucleotides sequence determinations revealed that all of the isolates obtained in 1994 and 1995, and 35 of 44 isolates from 2002 harbored *qacA*, while nine of the isolates from 2002 harbored *qacB*.

Relationship between PFGE banding patterns and chlorhexidine susceptibility

In 1990, there were only two MRSA isolates with the high chlorhexidine MIC of 4 µg/mL (both not belonging to type C, L, K, or M). All seven of the MRSA isolates with high chlorhexidine MIC obtained in 1994 belonged to PFGE type C. In 1995, 29 (96.7%) out of the 30 of MRSA isolates with high chlorhexidine MIC belonged to type C. In 2002, 40 isolates had high MICs of chlorhexidine, 14 of which (35%) belonged to type C, 11 (27.5%) belonged to type K, eight (20.0%) belonged to type M,

six (15%) belonged to type L, and one belonged to type N. The relationship between the PFGE banding patterns and chlorhexidine susceptibility is shown in Figure 2.

Relationship between PFGE banding patterns and *qacA/B* distribution

A total of 83 MRSA isolates collected in 1994, 1995, and 2002 were found to carry the *qacA/B* gene. Fifty-six isolates (75.7%) carrying the *qacA* gene belonged to PFGE type C, 11 isolates (14.9%) carrying the *qacA* gene belonged to type K and 6 isolates (8.1%) carrying the *qacA* gene belonged to type L. Only one isolate carrying the *qacA* gene belonged to type N. All isolates carrying the *qacB* gene belonged to PFGE type M. Figure 2 shows the relationship between PFGE banding patterns and the distribution of the *qacA/B* gene.

Relationship between the *qacA/B* gene and chlorhexidine susceptibility

In general, all of the MRSA isolates carrying the *qacA/B* gene had MICs equal to or higher than 2 µg/mL and 90.4% of them had MICs equal to or greater than the defined 'high' MIC. For those MRSA isolates that were devoid of the *qacA/B* gene, only 2.1% had high MICs. The relationship between *qacA/B* gene and chlorhexidine susceptibility is shown in Table 2.

Discussion

The present study demonstrates that the prevalence rate of MRSA isolates expressing high chlorhexidine MICs at the NTUH, a hospital using chlorhexidine as a major antiseptic, increased rapidly (from 3.3% to 66.7%) during the 13 years from 1990 through 2002. The prevalence rate of isolates collected in 2002 was similar to that reported from Japan (72%) (14).

Antiseptic resistant *S. aureus* is a global problem (12-16). An association between the intensity of chlorhexidine use and prevalence of micro-organisms with reduced susceptibility to chlorhexidine has been reported (25). To our best knowledge, however, our present study is the first study documenting the secular trend of chlorhexidine susceptibility in MRSA.

Chlorhexidine resistance in *S. aureus* is caused by a multidrug efflux pump in the bacterial cell membrane, which is encoded by two major groups of resistant genes, *qacA/B* and *smr* (14). The *qacA/B* gene confers a high-level resistance and is thus worthy of more concern clinically than the *smr* gene, which confers only a low-level resistance. Previous reports demonstrated that MRSA isolates carrying the *qacA/B* gene usually expresses chlorhexidine MIC levels equal to or higher than 4 µg/mL (15, 20, 21). Consistent with this, we presently observed that 90.4% of MRSA isolates carrying the *qacA/B* gene demonstrated chlorhexidine MICs matching or above the 4 µg/mL benchmark. Furthermore, nearly all our MRSA isolates that exhibited high chlorhexidine MICs (75/77, 97.4%) carried the *qacA/B* gene. Thus, we concluded that the high chlorhexidine MIC in the MRSA isolates in our institute was overwhelmingly due to *qacA/B*-mediated resistance. Our finding that the resistance was associated with the presence of *qacA* in 87% of these isolates differs from a report from Japan, which documented the presence of *qacB* in about 61% of the isolates (15). This genetic dichotomy, if indeed significant, remains to be clarified.

Prior to the present findings, the clinical significance of elevated chlorhexidine MICs in MRSA has been contentious. Although some authors proposed that chlorhexidine appeared to be as effective as a hand-washing agent for MRSA regardless of the MIC levels and thus the phenomenon of elevated chlorhexidine MIC levels in MRSA should not be interpreted as clinical resistance (20). However, others reported that hand disinfectants containing only chlorhexidine (e.g., Hibiscrub) were less effective against MRSA isolates with high chlorhexidine MIC levels, and disinfectants containing both alcohol and chlorhexidine (e.g., Hibisol) were more preferred (17). In addition, some studies published recently has demonstrates that alcohol-based hand rubs, even without the presence of chlorhexidine, were more effective than chlorhexidine-based products in terms of spectrum of activities and rapidness of killing (26, 27). Chlorhexidine-based products were noted more easily to

lead to dryness of skin, impairment of skin barrier, skin irritation, and allergic reaction than alcohol-based hand rubs (26). It had also been demonstrated that the acceptance of using alcohol-based hand rubs by HCWs was better than using chlorhexidine-based disinfectants, which in turn increased the adherence of HCWs to hand hygiene (27). Taking all above factors into consideration, it is suggested to consider changing the antiseptic policy at a hospital with a high prevalence of chlorhexidine-resistant pathogens, such as NTUH, from using chlorhexidine-based hand disinfectant to alcohol-based hand rubs.

Type C MRSA isolates appeared at the NTUH in 1994 and became the predominant strain by 1995 (22). The differences of banding patterns between type C and type K, and L were less than six bands (Figure 1). According to the report of Tenover et al, the banding patterns of PFGE of MRSA isolates originating from a single clone might vary by up to six bands even over a period of only six months (28). In addition, MRSA isolates belonging to PFGE types K and L express the same pattern of antibiotics resistance as those of type C (data not shown). Therefore, MRSA isolates belonging to PFGE types K and L could be considered as belonging to subtypes of type C, with their greater genetic variation being explained by a more prolonged evolution time.

Analyzed in this way, 77.5% (31/40) of our MRSA isolates with high chlorhexidine MICs in 2002 belonged to the same PFGE type (Type C). Therefore, nearly all of the MRSA isolates with high chlorhexidine MIC levels originated from PFGE type C (type K and L were considered as subtypes of type C) (67/77, 87.0%), with the majority harboring *qacA* gene (Figure 2). The relationship between the prevalence rate of MRSA isolates with high chlorhexidine MIC and the prevalence rate of type C MRSA in four different years are shown in Figure 3.

As stated above, following their detection at the NTUH in 1994, the type C MRSA isolates had become predominant only one year later (22). Before the era of type C MRSA isolates, the prevalence rate of MRSA isolates with high chlorhexidine MIC levels was only 3.3% at the same hospital. All of the type C MRSA isolates in 1994 carried the *qacA* gene and expressed high chlorhexidine MIC levels (Figure 2). Therefore, the possibility that type C MRSA isolates acquired the *qacA* gene from other bacteria after their introduction into the NTUH is very low. It is much more likely that type C MRSA harbored the *qacA* gene and expressed high chlorhexidine MIC levels before they were introduced into the NTUH. In our previous study, type C MRSA isolates were also demonstrated to be highly resistant to many antibiotics and only susceptible to glycopeptide and rifampin (10). Because of their resistance to multiple antibiotics and expression of high chlorhexidine MIC levels, type C MRSA isolates more easily survived than other MRSA strains under the specific selective

pressure in this hospital where chlorhexidine and antibiotics were widely used. Once introduced into this hospital, type C MRSA rapidly became the predominant MRSA strain and the prevalence rate of MRSA isolates with high chlorhexidine MIC levels increased rapidly.

MRSA isolates that belonged to the PFGE type M, which were first noted in 2002, were also interesting. They carried the *qacB* gene and also expressed chlorhexidine resistance. They were additionally resistant to erythromycin, clindamycin, and fluoroquinolones but had been shown to be susceptible to trimethoprim/sulfamethoxazole, rifampin, tetracycline, and glycopeptides. Although they are more susceptible to some antimicrobial agents than type C strains, the possibility of their emergence as a significant problem cannot be discount.

In conclusion, the present study demonstrates that the prevalence rate of MRSA isolates with high chlorhexidine MICs at the NTUH has increased rapidly from 1990 to 2002. The introduction and rapid predominance of an endemic MRSA strain carrying the *qacA* gene and expressing high chlorhexidine MICs as well as multi-antibiotic resistance may partially explain why a rapid increase of MRSA isolates with high chlorhexidine MICs has been observed in the NTUH. With the consideration of decrease of chlorhexidine susceptibility in MRSA isolates, the shortage of chlorhexidine-based product in spectrum of activities and toxicity to human skin, and the benefits of alcohol-based hand rubs, the antiseptic policies at a hospital with a high percentage of chlorhexidine resistance in some important pathogens, such as NTUH, maybe need changed.

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Biography of first author

Dr. Jann-Tay Wang is an attending staff with specialty in infectious diseases at National Taiwan University Hospital.

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Table 1. The susceptibility of 240 MRSA isolates to chlorhexidine, stratified by the year of isolation.

Year of isolation (No. of isolates)	No. of isolates with different MIC* levels ($\mu\text{g/mL}$) (%)					MIC ₉₀ ($\mu\text{g/mL}$)
	0.5	1	2	4	8	
1990 (60)	0	27 (45%)	31 (51.7%)	2 (3.3%)	0	2
1994 (60)	0	1 (1.7%)	52 (86.7%)	1 (1.1%)	6 (10%)	4
1995 (60)	2 (3.3%)	10 (16.7%)	18 (30%)	24 (40%)	6 (10%)	4
2002 (60)	0	2 (3.3%)	18 (30%)	27 (45%)	13 (21.7%)	8

*minimum inhibitory concentration

Table 2. Correlation between *qacA/B* gene and chlorhexidine resistance in 240 MRSA isolates isolated in 1990, 1994, 1995, and 2002 at NTUH.

Isolates with or without <i>qacA/B</i> gene (No.)	No. of isolates with different MICs ($\mu\text{g/mL}$) (%)				
	0.5	1	2	4	8
With <i>qacA</i> gene (74)	0	0	7 (9.5%)	43 (58.1%)	24 (32.4%)
1990 (0)	0	0	0	0	0
1994 (7)	0	0	0	1(14.3%)	6 (85.7%)
1995 (32)	0	0	3 (9.4%)	23 (71.9%)	6 (18.8%)
2002 (35)	0	0	4 (11.4%)	19 (54.3%)	12 (34.3%)
With <i>qacB</i> gene (9)	0	0	1 (11.1%)	8 (88.9%)	0
1990 (0)	0	0	0	0	0
1994 (0)	0	0	0	0	0
1995 (0)	0	0	0	0	0
2002 (9)	0	0	1 (11.1%)	8 (88.9%)	0
Without <i>qacA/B</i> gene (157)	2 (1.3%)	40 (25.5%)	111 (70.7%)	3 (1.9%)	1 (0.6%)
1990 (60)	0	27 (45%)	31 (51.7%)	2 (3.3%)	0
1994 (53)	0	1 (1.9%)	52 (98.1%)	0	0
1995 (28)	2 (7.1%)	10 (35.7)	15 (53.6%)	1 (3.6%)	0
2002 (16)	0	2 (12.5%)	13 (81.3%)	0	1 (6.3%)

Figure 1

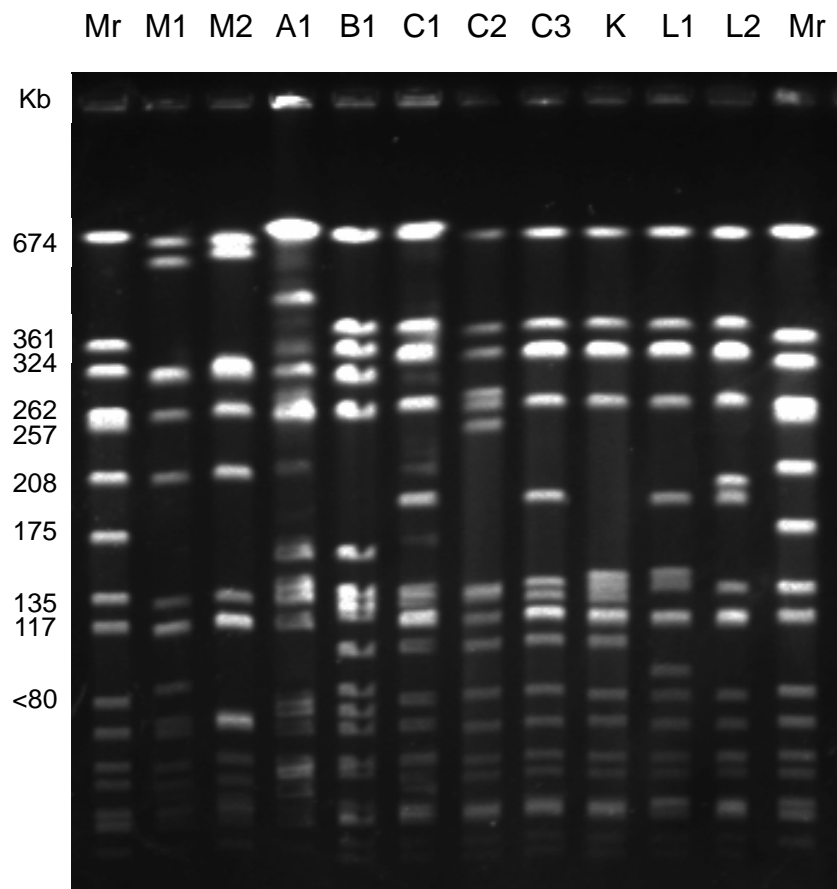


Figure 2A



Figure 2B

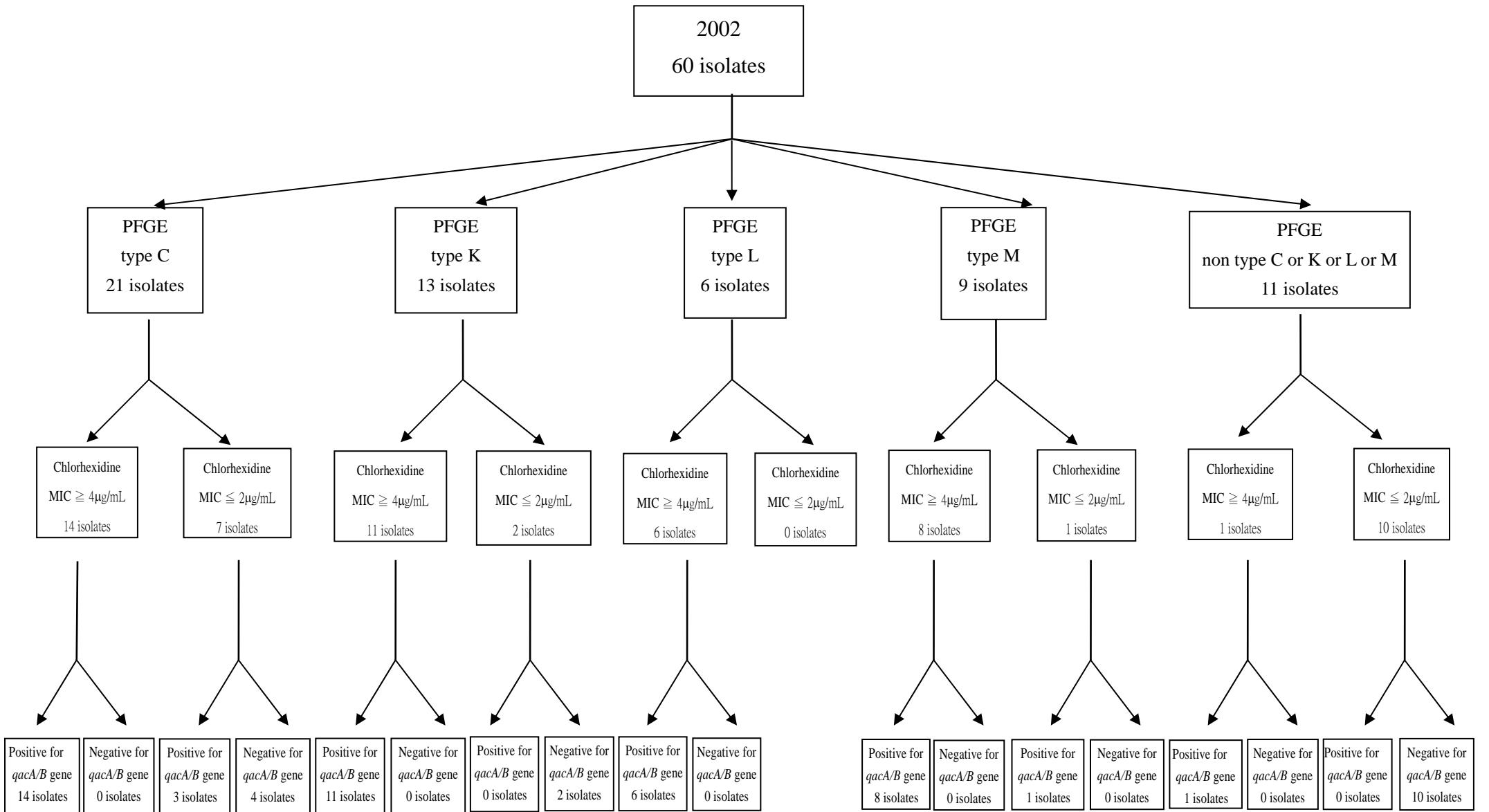
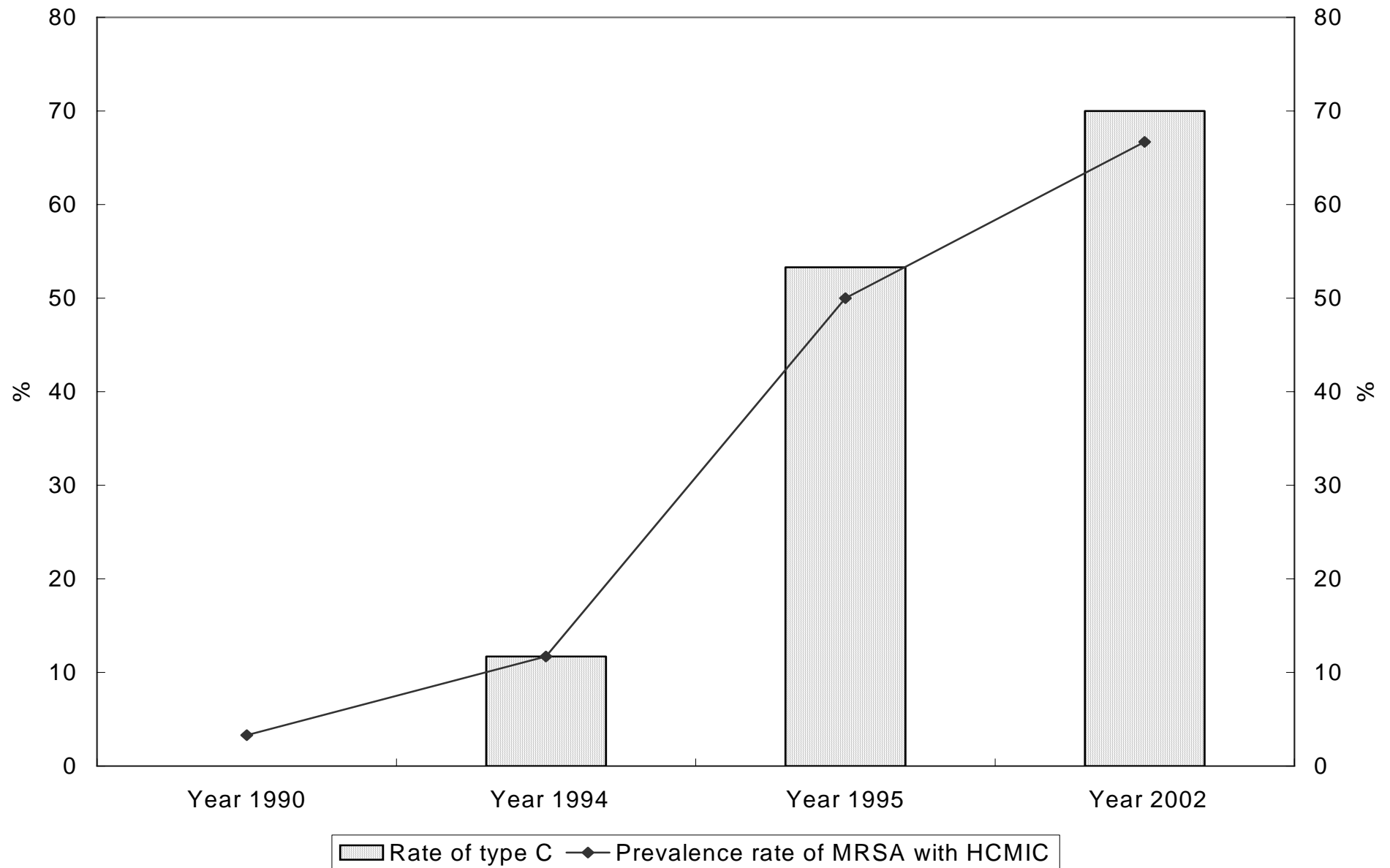


Figure 3



Legends of figures

Figure 1. Pulsed-field gel electrophoresis banding patterns of type M1, M2, A1, B1, C1, C2, C3, K, L1, and L2 MRSA isolates. Lane Mr: molecular weight marker (*Staphylococcus aureus* NCTC 8325).

Figure 2. (A) The relationship between pulsed-field gel electrophoresis banding patterns, chlorhexidine MIC, and distribution of *qacA/B* gene in MRSA isolates obtained in 1990, 1994, and 1995 at the NTUH. (B) Relationship between pulsed-field gel electrophoresis banding patterns, chlorhexidine MIC, and distribution of *qacA/B* gene in MRSA isolates obtained in 2002 at the NTUH.

Figure 3. Prevalence rate of type C MRSA isolates and MRSA isolates with high chlorhexidine MIC at the NTUH. In 2002, the types K and L were also considered as subtypes of type C. Abbreviation: HCMIC, high chlorhexidine minimum inhibitory concentration.