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ORIGINAL ARTICLE

Antimicrobial resistance of *Moraxella catarrhalis* isolates in Taiwan

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Background/Purpose: The prevalence of ampicillin-resistant *Moraxella catarrhalis* has been higher in Taiwan than in other countries, with reports of 97.7% in the 1990s. The aims of this study were to assess resistance trends for *M. catarrhalis*, which causes respiratory tract infections, against several classes of oral antibiotics and to compare the minimum inhibitory concentration (MIC) of antimicrobial agents against *M. catarrhalis* isolates between 1993–1994 and 2001–2004.

Methods: Clinical isolates of *M. catarrhalis* ($n = 314$) were collected from 11 large medical centers in Taiwan between 2001 and 2004. β-Lactamase production tests were performed. The MICs for 13 different oral antibiotics were calculated using the agar dilution method. Pulsed-field gel electrophoresis (PFGE) was performed for 18 randomly selected high-level ampicillin-resistant (BRO-1 β-lactamase-positive, MIC $\geq$ 32 μg/mL) isolates to investigate their genetic relatedness.

Results: The overall rate of β-lactamase-producing isolates was 97.8% (307/314). All isolates were susceptible to amoxicillin + clavulanate, chloramphenicol, cefixime, ciprofloxacin, erythromycin, levofloxacin, moxifloxacin, and roxithromycin. The rate of resistance to cefaclor and cefuroxime was 8.3% and 1.3%, respectively, while no resistance was found in 1993–1994. Resistance to trimethoprim–sulfamethoxazole (SXT) and tetracycline was 18.5% and 19.8%, respectively. Comparison of 1993–1994 and 2001–2004 isolates revealed that the zone diameter for amoxicillin + clavulanate disks decreased from 43 mm in 1993–1994 to 32 mm in 2001–2004 ($p < 0.001$). However, MIC_{50} (0.25 μg/mL in both 1993–1994 and 2001–2004) and MIC_{90} (0.5 μg/mL in both 1993–1994 and 2001–2004) for amoxicillin + clavulanate did not differ

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between the study periods. The PFGE typing results demonstrate that at least two closely related BRO-1 clones are spreading in Taiwan.
Conclusion: The rates of resistance to cefaclor, cefuroxime, tetracycline and SXT are now increasing in Taiwan. Molecular typing showed that at least two closely related BRO-1 clones are circulating. Although amoxicillin + clavulanate remains the antimicrobial therapy of choice for *M. catarrhalis* infections, continued surveillance of antimicrobial susceptibility and application of control measures against further transmission are required to inhibit the emergence of the resistant strains.

Introduction

Moraxella catarrhalis (formerly *Branhamella catarrhalis*), a Gram-negative aerobic diplococcus, has been recognized as an increasingly important pathogen in respiratory infections including otitis media, sinusitis, acute bronchitis, and pneumonia.[1,2] It resides exclusively in humans and colonizes the nasopharynx and occasionally the conjunctiva and genital tract.[3] Colonization of the respiratory tract is believed to be a precursor of infection, although the mechanism is not well understood.[4] A high percentage (up to 75%) of colonization was found in infants and decreased with age.[3] The prevalence of colonization in healthy adults is low (1–3%).[5]

M. catarrhalis isolates frequently harbor a β-lactamase enzyme designated BRO (from *Branhamella* and *Moraxella*). Two distinct BRO-type β-lactamase enzymes, BRO-1 and BRO-2, have been found since 1976.[6] BRO-positive isolates have increased rapidly in recent years, and account for more than 80–90% of isolates in Europe and North America[7,8] and nearly 98% in Taiwan.[9] Although a third BRO enzyme (BRO-3) was postulated by Christensen et al,[10] BRO-3 is now considered to be a membrane-bound precursor rather than a distinct enzyme.[11] BRO-1 and BRO-2 can be detected by polymerase chain reaction (PCR) methods using specific primers.[12] Our previous investigation revealed that BRO-1 is the most common enzyme among β-lactamase-positive isolates (238/270 isolates, 88%), with only 12% (32/270) of β-lactamase-producing isolates containing BRO-2.[13] Both enzymes are encoded by chromosomal genes and are phenotypically identical and membrane-associated. The proteins differ by only a single amino acid.[14] Fung et al showed significant differences between the geometric mean minimum inhibitory concentration (MIC) of ampicillin for BRO-1- and BRO-2-producing strains, and smaller differences for amoxicillin + clavulanate, loracarbef, cefixime, and cefetamet.[15] The difference is attributed to the production of more enzymes as a consequence of the higher transcriptional activity of the *BRO-1* gene.[4,6]

The continuing increase in the antibiotic resistance of respiratory pathogens remains a global problem. Surveillance to monitor shifting trends in resistance is vital and ultimately influences the selection of antimicrobial agents available for use against a particular organism.[16] Jaecklin et al emphasized the importance of ongoing antimicrobial susceptibility surveillance studies at a regional level.[17]

In this study, we collected 314 *M. catarrhalis* isolates from 11 medical centers in Taiwan in 2001–2004 to assess resistance trends for this organism against 13 different oral antibiotics used to treat respiratory tract infections. The rapid increase in β-lactamase-producing strains of *M. catarrhalis* is a global problem. The ultimate aim of the study was to obtain a clearer understanding of BRO distributions to gain an insight into clonal spreading in Taiwan. Therefore, we compared MIC values to those of isolates obtained in 1993–1994 and determined the genetic relatedness of ampicillin-resistant isolates using pulsed-field gel electrophoresis (PFGE).

Material and Methods

Bacterial strains

A total of 314 clinical isolates of *M. catarrhalis* were consecutively collected from 11 geographically scattered laboratories in Taiwan in 2001–2004. One isolate was accepted for each episode of infection. The isolates were sent to Taipei Veterans General Hospital for further identification and antimicrobial susceptibility testing. Organisms were immediately subcultured onto chocolate agar plates to check viability and purity. Identification was confirmed by Gram stain, colony morphology, hydrolysis of tributyrin, and positive oxidase and DNase tests.[15] β-Lactamase production was confirmed by a nitrocefin test.[15]

Antimicrobial susceptibility testing

To assess the trends of ampicillin resistance of *M. catarrhalis*, isolates were inoculated into 1 mL of brain–heart infusion (BHI) broth with 5% Fildes' extract supplement and incubated for 5 h at 37°C. Each suspension was diluted 1:100 in peptone water and swabbed onto Muller-Hinton (MHA) agar supplemented with 0.25% lysed horse blood. Disks of 6 mm in diameter containing ampicillin (2 μg) or amoxicillin + clavulanate (2 μg + 1 μg) were applied. All plates were incubated in 5% CO_2 and 95% air at 37°C for 18 h. Zone diameters were measured and recorded to the nearest whole millimeter.

MIC values for the 13 antimicrobial agents were determined using an agar dilution technique with horse-blood-supplemented MHA agar. A Denley multipoint inoculator (Denley Instruments, Billingshurst, UK) was used to deliver 0.003 mL of 1:100 dilutions of each organism prepared as for disk testing (10^4 colony-forming units/spot) to the agar surface. All plates were incubated in 5% CO_2 and 95% air at

37°C for 18 h. MIC was defined as the lowest concentration of an antimicrobial agent that inhibited visible growth. MIC_{50} and MIC_{90} were defined as the minimal concentration that inhibited 50% and 90% of bacterial growth, respectively. The interpretation of cut-off point for resistance was based on Clinical Laboratory Standards Institute (CLSI) recommendations for *Haemophilus* spp.[18] When CLSI breakpoints were not available, The British Society for Antimicrobial Chemotherapy (BSAC) breakpoints for erythromycin, ciprofloxacin, levofloxacin, and moxifloxacin and PK/PD breakpoints for cefixime were used.[19] For roxithromycin, we used the resistance criterion (MIC $\geq$ 32 μg/mL) established by Abdel-Rahman et al.[20]

Staphylococcus aureus ATCC 29213, *Escherichia coli* 35218, *M. catarrhalis* ATCC25238 (β-lactamase-negative) and *M. catarrhalis* Ravasio (BRO-1) were used as control strains. Media, supplements and disks were BD-Difco products purchased from Becton Dickenson (Franklin Lakes, NJ, USA). Standards were obtained from the following manufacturers: ampicillin, chloramphenicol, erythromycin, and tetracycline from Sigma-Aldrich (St. Louis, MO, USA); amoxicillin + clavulanate from GlaxoSmithKline (London, UK); cefaclor and cefuroxime from Eli Lilly (Indianapolis, IN, USA); cefixime from Astellas Pharmaceutical (Tokyo, Japan); levofloxacin from Daiichi Sankyo Company (Tokyo, Japan); moxifloxacin from Bayer AG (Leverkusen, Germany); roxithromycin from Hoffmann Le Roche (Basel, Switzerland) and trimethroprim–sulfamethoxazole (SXT) from Shionogi (Osaka, Japan).

Pulsed field gel electrophoresis

We randomly selected 18 high-level ampicillin-resistant (MIC $\geq$ 32 μg/mL) *M. catarrhalis* isolates from different regions of Taiwan (isolates 1–6 from the northern region, 7–12 from the central region, and 13–18 from the southern region). It was confirmed that these 18 isolates were BRO-1 β-lactamase-producing strains by isoelectric focusing (IEF).[13] The selected isolates were inoculated into supplemented tryptic soy broth (TSB) and incubated with shaking for 18 h at 37°C. For restriction endonuclease digestion, an agarose plug containing DNA was placed into restriction buffer containing 12 U of *Spe*I (New England Biolabs, Beverly, MA, USA) and incubated overnight at 37°C. Lambda concatamers (Lambda Ladder PFG marker; New England Biolabs) were used as molecular weight standards. Electrophoresis was performed on a contour-clamped homogeneous gel electrophoresis device (CHEF mapper XA system; Bio-Rad, Hercules, CA, USA). After staining with ethidium bromide, the gel was photographed with UV transillumination, and restriction fragment length polymorphism (RFLP) patterns were determined by visual inspection of the gels. The band patterns were compared as previously described by Tenover et al.[21] A dendrogram was generated with GelCompar II statistical software (Applied Maths NV, St-Martens-Latem, Belgium) using the Dice coefficient and unweighted paired-group methods for the arithmetic average algorithm.

Statistical analysis

Statistical analysis was performed using SPSS statistical software Version 17.0 (SPSS, Chicago, IL, USA). Data for zone diameter, MIC and antibiotic resistance rates between 1993–1994 and 2001–2004 were compared using the Mantel–Haenszel test, the Mann–Whitney *U* test, and Fisher's exact test, respectively. A p value <0.05 was considered as statistically significant.

Results

A total of 314 isolates from 11 medical centers in different regions of Taiwan were collected in 2001–2004. Of these, 124 isolates (39.4%) were from northern Taiwan, 98 isolates (31.2%) were from central Taiwan, 77 isolates (24.5%) were from southern Taiwan, and 15 isolates (4.8%) were from eastern Taiwan. The age of patients from whom the isolates were obtained ranged from 1 to 96 years (mean 38.4 years). The ratio of males to females was 1.57:1. Among the 314 isolates, 176 (57%) were from sputum, 58 (18.5%) from nasal or sinus specimens, 12 from wound, nine from throat, three from blood, three from ear, two from eye and one from endometrium samples.

All β-lactamase-producing *M. catarrhalis* isolates were ampicillin-resistant. The zonal diameter for *M. catarrhalis* was 6–28 mm for ampicillin and 25–50 mm for amoxicillin + clavulanate. Only seven isolates were susceptible to ampicillin and the overall percentage of β-lactamase-producing isolates was 97.8%. The mean zone diameter was 32 mm for amoxicillin + clavulanate, which was significantly different from the 15 mm for ampicillin. Comparison of data for 1993–1994[13] and 2001–2004 (Fig. 1) revealed that the mean zone diameter for amoxicillin + clavulanate decreased from 43 mm in 1993–1994 to 32 mm in 2001–2004 ($p < 0.001$). However, MIC_{50} (0.25 μg/mL in both 1993–1994 and 2001–2004) and MIC_{90} (0.5 μg/mL in both 1993–1994 and 2001–2004) for amoxicillin + clavulanate did not change between the study periods.

All isolates were susceptible to amoxicillin + clavulanate, chloramphenicol, cefexime, ciprofloxacin, erythromycin,

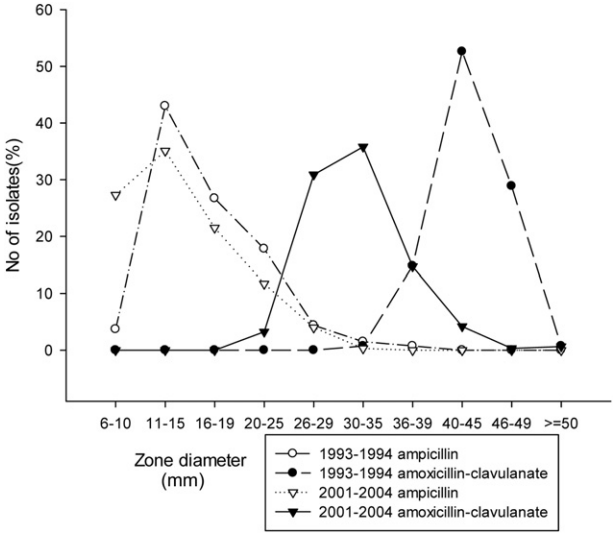

Figure 1. Comparison of zone diameters (mm) for ampicillin and amoxicillin + clavulanate between 1993–1994 (n = 124) and 2001–2004 (n = 314).

levofloxacin, moxifloxacin and roxithromycin. Furthermore, 26 isolates (8.3%) were resistant to cefaclor, four (1.3%) were resistant to cefuroxime, 58 (18.5%) were resistant to SXT, and 62 (19.5%) were resistant to tetracycline (Table 1). Among the isolates from different regions, the rate of resistance to SXT was 8.1% in the northern region, 32.7% in the central region, and 14.3% in the southern region ($p < 0.001$ central vs north, $p < 0.01$ south vs central). The rate of resistance to tetracycline was 15.3%, 32.7%, and 16.9% in the northern, central, and southern regions, respectively ($p < 0.005$ central vs north, $p < 0.05$ south vs central; Fig. 2). To investigate the possibility of BRO-1 clonal spreading, a total of 18 high-level ampicillin-resistant isolates (BRO-1-positive, MIC $\geq$ 32 μg/mL) randomly selected from different geographical regions were analyzed by PFGE. The resulting dendrogram shows diverse group clusters in *Spe*I-digested PFGE patterns of isolates (Fig. 3). Isolates 2 (from the northern region) and 8 (from central region) had closely related banding patterns, and isolates 11 (from the central region) and 18 (from the southern region) had closely related PFGE profiles. Genomic typing demonstrated that at least two closely related M. catarrhalis clones had been transmitted in three regions of Taiwan, which indicates that high-level ampicillin-resistant strains are spreading in the country.

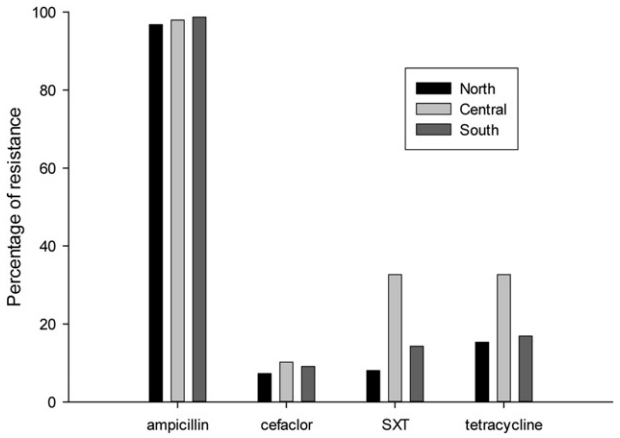

Figure 2. Rate of resistance to ampicillin, cefaclor, trimethoprim + sulfamethoxazole (SXT) and tetracycline for *M. catarrhalis* isolates from northern, central and southern Taiwan (2001–2004). SXT, $p < 0.001$ for central versus north and $p < 0.01$ for south versus central. Tetracycline, $p < 0.005$ for central versus north and $p < 0.05$ for south versus central.

Disscussion

M. catarrhalis, previously considered a harmless commensal organism, has gained recognition as a pathogen over the past three decades and has shown increased prevalence of β-lactamase production. Before 1976, there were no reports of β-lactamase-producing strains of *M. catarrhalis*.[6] However, a study using isolates obtained in the late 1980s from the UK revealed that 90% were β-lactamase producers.[7,22] In Taiwan, the prevalence of ampicillin-resistant *M. catarrhalis* is high, with a report of 97.7% in 1994.[9] The present finding (97.8% β-lactamase producers) is compatible with previous studies.[9,23] The dramatic increase in the frequency of β-lactamase-producing *M. catarrhalis* strains could be regarded as the fastest dissemination of any known β-lactamase within a bacterial species.[6,23]

The well-known mechanism of aminopenicillin resistance in *M. catarrhalis* involves the expression of one of two chromosomally encoded BRO β-lactamases, detectable

Table 1 Comparative *in vitro* activity of 13 antimicrobial agents against 314 *M. catarrhalis* isolates

Antimicrobial agent	MIC in 2001–2004 (μg/mL)			Resistance (%)[a]	
	Range	MIC_{50}	MIC_{90}	2001–2004	1993–1994
Ampicillin	0.008–64	8	32	97.8	97.7
Amoxicillin + clavulanate	0.008–0.5	0.25	0.5	0	0
Chloramphenicol	0.25–1	0.5	1	0	0
Cefaclor	0.25–128	2	16	8.3*	0
Cefuroxime	0.25–16	2	4	1.3	0
Ciprofloxacin	0.015–2	0.06	0.5	0	0
Cefixime	0.03–1	0.25	0.5	0	0
Erythromycin	0.12–2	0.25	1	0	0
Levofloxacin	0.06–4	0.06	1	0	N
Moxifloxacin	0.06–2	0.12	0.25	0	N
Roxithromycin	0.12–4	0.5	2	0	0
Trimethoprim + sulfamethoxazole	0.12–8	0.5	8	18.5	100/9.6[b]
Tetracycline	0.25–128	0.5	64	19.8	14

[a] CLSI breakpoints for resistance: ampicillin ≥4 μg/mL, amoxicillin + clavulanate (2:1 ratio) ≥8/4 μg/mL, chloramphenicol ≥8 μg/mL, cefaclor ≥32 μg/mL, cefuroxime ≥16 μg/mL, tetracycline ≥8 μg/mL, trimethoprim–sulfamethoxazole(1:19 ratio) ≥4/76 μg/mL. BSAC breakpoints for resistance: erythromycin ≥16 μg/mL, ciprofloxacin ≥1 μg/mL, levofloxacin ≥2 μg/mL, moxifloxacin ≥1 μg/mL. PK/PD breakpoint for resistance: cefixime ≥2 μg/mL. Breakpoint for resistance: roxithromycin ≥32 μg/mL.[18]

[b] TMP 100% resistant, SMX 9.6% resistant.

*Significant increase in resistance compared with 1994 ($p < 0.05$).

MIC = minimum inhibitory concentration; N = test not performed in 1993–1994.

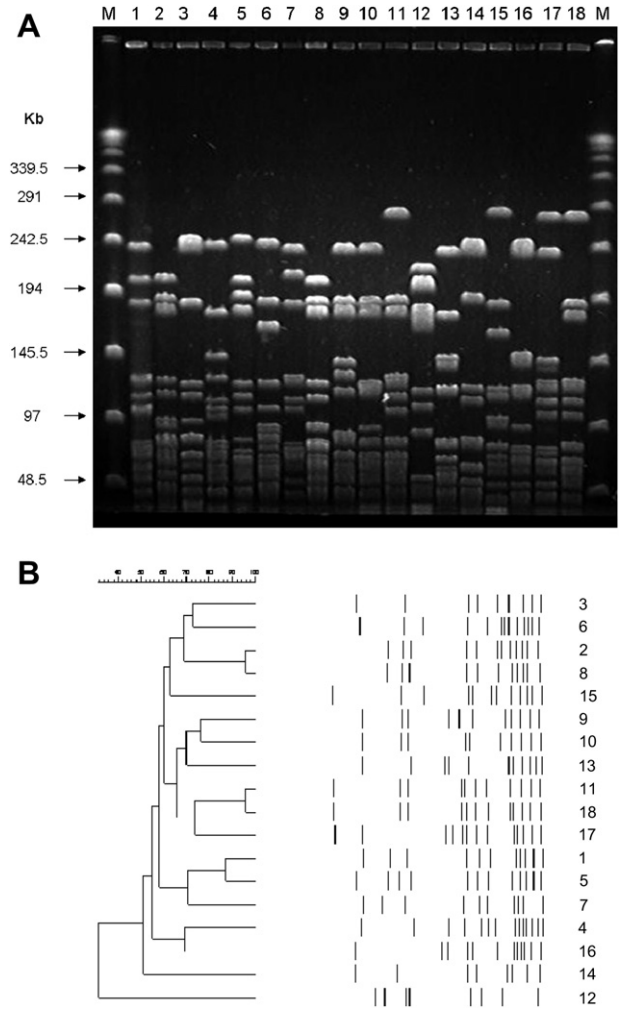

Figure 3. Pulsed-field gel electrophoresis profiles for 18 high-level ampicillin-resistant *M. catarrhalis* isolates. (A) DNA fragments were digested using *Spe*I restriction endonuclease. Lane M = molecular weight marker; lanes 1–6 = isolates from northern Taiwan; lanes 7–12 = isolates from central Taiwan; lanes 13–18 = isolates from southern Taiwan. (B) Dendrogram showing clustering of *Spe*I-digested PFGE patterns for isolates.

in more than 90% of clinical isolates.[2] One study reported a significant increase in MIC for ampicillin and amoxicillin in M35 porin-deficient mutants,[24] which suggests that this porin affects outer membrane permeability for aminopenicillins in a clinically relevant manner.

The mean zone diameter for amoxicillin + clavulanate decreased from 43 mm in 1993–1994 to 32 mm in 2001–2004. However, the MIC_{90} value in 2001–2004 (0.5 μg/mL) was the same as that in 1993–1994. The reason for the change in zone diameter is not clear, but it is possible that the proportion of BRO-1- and BRO-2-producing isolates changed. We did not perform analytical IEF or PCR for BRO-1 and BRO-2 detection for all ampicillin-resistant isolates, so we could not calculate the percentage of BRO-1 and BRO-2 isolates. According to our previous study, the geometric mean amoxicillin + clavulanate MIC was threefold higher for BRO-1 producers than for BRO-2 producers.[13] Therefore, if the proportion of BRO-1 isolates were increasing, the zone diameter for amoxicillin + clavulanate would decrease.

In present study, activity based on the MIC_{90} of cephalosporin was ranked in the order cefixime (0.5 μg/ml) > cefuroxime (4 μg/ml) > cefaclor (16 μg/ml). The rate of resistance to cefaclor and cefuroxime was 8.3% and 1.3%, respectively, while no resistance was found in 1993–1994. Interestingly, comparison of data between 1993–1994 and 2001–2004 revealed an increase in MIC_{90} for cefaclor (1 μg/mL in 1993–1994 vs 16 μg/mL in 2001–2004, $p < 0.01$) and cefuroxime (2 μg/mL in 1993–1994 vs 4 μg/mL in 2001–2004, $p < 0.01$), which is consistent with the significant increase in resistance to cefaclor (8.28% in 2004, 0% in 1993–1994, $p < 0.05$). Kadry et al suggested that BRO β-lactamases might give some protection against many of the newer cephems.[25] An investigation of trends in antibiotic resistance of 375 *M. catarrhalis* isolates in a single hospital over a 10-year period (1984–1994) revealed the increased MIC was not due to an increase in the frequency of β-lactamase-producing strains, but rather occurred mainly within the group of β-lactamase-positive strains.[26] The same phenomenon was also found for tetracycline (MIC_{90}16 μg/mL in 1993–1994 vs 64 μg/mL in 2001–2004, $p < 0.01$), which suggests that continual monitoring of *M. catarrhalis* resistance to these antibiotics is necessary. The rate of resistance to SXT was 18.5%, which could not be compared with results for trimethoprim (100%) and sulfamethoxazole (9.6%) in 1993–1994 owing to the combination (1:19 ratio) used in this study. However, The SENTRY surveillance program in 1997–1999 found a rate of resistance to SXT of only 0.1%–2.6%[27] and Karpanoja et al found a rate of 3.2–14.5%.[28] Compared with the data in recent studies, the rate of resistance to SXT is higher in Taiwan.[23,27,28] Our isolates were geographically dispersed and the rate of resistance to SXT was higher in the central region (32.7%) than in northern (8.1%) and southern (14.3%) regions of Taiwan. The reason for this increase in SXT resistance is not clear. Many studies have provided evidence of a connection between increased consumption and increased resistance to certain antimicrobial agents.[29,30] However, a study that collected 14,138 *M. catarrhalis* isolates between 1998 and 2004 found no statistically significant association between regional SXT use and resistance in *M. catarrhalis*.[28]

In this study, PFGE analysis was carried out for high-level ampicillin-resistant (MIC ≥ 32 μg/mL) isolates confirmed as BRO-1-producing strains from different regions of Taiwan. Although the selected number was small (18/307, 5.9%), we found two closely related BRO-1 clones in northern, central and southern regions, which indicates that these clones are spreading in Taiwan. According to a report from Japan, *M. catarrhalis* can be transmitted from patient to patient.[31] Preventive measures such as hand-washing and mask and glove protection for healthcare workers should be adopted to control hospital outbreaks.

In conclusion, the rates of resistance to cefaclor, cefuroxime, tetracycline and SXT are now increasing in Taiwan, Molecular typing showed that at least two closely related BRO-1 clones are circulating. Although amoxicillin + clavulanate remains the antimicrobial therapy of choice for *M. catarrhalis* infections, continued surveillance of antimicrobial susceptibility and application of control measures

against further transmission are required to decrease the emergence of the resistant strains.

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