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Identification and Differences in Antimicrobial Susceptibility of *Lactococcus garvieae* from Farmed Grey Mullet (*Mugil cephalus*) and non-Grey Mullet in Southern Taiwan

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Abstract

Streptococcal infection is a main infectious diseases for farmed grey mullet (*Mugil cephalus*). This study were to identify spreptococcal species in diseased farmed grey mullet and to investigate differences in susceptibility to 13 antibiotics and in genotypes between the stains from the grey mullet and non-grey mullet. 170 samples from diseased farmed grey mullet were collected from three county in 2013 -2016. Multiplex PCR identified *L. garviea* (146) as the main pathogen, *S. agalactia* (9), *S. dysgalactiae* (19), and double infection (5), but no *S. iniae*. The prevalence changed annually and differed among three counties. Pulsed-field gel electrophoresis (PFGE) analysis demonstrated identical genotype with an *Apa*I-digested DNA pattern. Disc diffusion results demonstrated differences in antibiotic susceptibility between the strains from grey mullet (146) and non-grey mullet (30). Almost all strains resisted to clindamycin and all strains were susceptible to six antibiotic in grey mullet and 4 antibiotics in non-grey mullet. The reduced susceptible strains was more in non-grey mullet than grey mullet group. The reduced susceptible strains were observed the highest in 2014 and in Chiayi county and decreased from 2014 to 2016. However, the strains with reduced susceptibility to ceftriaxone, cirpofoxacin, moxifloxacin, tetracycline for human treatment were observed.

Keywords: *Lactococcus garvieae;* Grey Mullet (*Mugil cephalus*);; Multiplex PCR; Antibiotic Susceptibility

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1. Introduction

Grey mullet (*Mugil cephalus* Linnaeus) is a main farmed fish species with high economic value in Southern Taiwan. These fish are mainly farmed for their mullet roe, which is a type of food. In a cultured environment, diseases are key determinants of whether grey mullet can be harvested. A study investigating diseases in brood grey mullet during the cultivation period revealed that streptococcosis is one of the major bacterial diseases[1]. However, not all of the Gram-positive streptococcus isolated in that study were *Streptococcus* spp. Therefore, further identification was required [2]. Of course, studies conducted in different years are crucial references for strain infection epidemiology. Streptococcal infection is a threat worldwide. Earlier studies have been unable to identify the strain down to the species level[3,4]. Currently, at least 14 types of pathogenic Gram-positive streptococcus, namely *Enterococcus faecalis* (syn. *S. faecalis*), *S. agalactiae* (syn. *S. difficile*), *S. castoreus*, *S. dysgalactiae, S. halichoeri*, *S. iniae* (syn. *S. shiloi*), *S. marimammalium*, *S. milleri*, *S. parauberis*, *S. phocae*, *L. garvieae* (syn. *E. seriolicida*), *L. piscium*, *L. lactis*, and *Vagococcus salmoninarum* are known to be present in fish [5,6,7,8]. Zlotkin *et al*. designed *L. garvieaea* specific primer pairs that could distinguish and identify *L. lactis*, *S. iniae*, and *Aeromonas salmonicida*, and the expected amplification product was 1100 bp [9]. Mata *et al*. used multiplex polymerase chain reaction (PCR) to analyze streptococcosis, and were able to simultaneously diagnose infections from four different strains, namely *S. iniae*, *S. difficilis*, *S. parauberis*, and *L. garvieae* [10]. In addition to identifying strains, studies have also adopted technological approaches such as serotyping, biotyping, and pulsed-field gel electrophoresis (PFGE) for molecular typing to study phylogenetics in epidemiology [11,12,13,14.15,16]. Clinically, because Gram-positive streptococcal infection is very common, medicine susceptibility testing for antibiotics treatment is critical for treating fish diseases on site [17,18,19,20]. The public health problem of antibiotic resistance has attracted scholars' attention [21]. The objectives of this study were to establish a method for the rapid diagnosis of Gram-positive streptococcal infection in cultured grey mullet and to clarify the differences between infectious strains by using the results of studies conducted in different years to provide a foundation for subsequent vaccine research. Problems with medical treatment for the main infection strains encountered on site can be identified using the results of antibiotic susceptibility testing conducted in this study. We can also use these results to determine whether fish exhibit resistance to antibiotics not developed for use on fish, which would serve as a warning sign.

2. Materials and Methods

2.1 Sample collection

A total of 170 samples of the heart, liver, kidneys, spleen, and brain were collected from diseased farmed grey mullet from three counties Yunlin, Chiayi, and Tainan in southern Taiwan from 2013 to 2016. The samples were ground and plated on blood agar (BAP, Difco) containing 5% defibrinated sheep blood. The plates were placed for bacterial growth at 28°C for 24–72 h. This experiment also included 36 *L. garvieae* strains from other fish species. Bacterial species were examined by Gram-staining, catalase testing, the Rapid ID 32 STREP system (Bio-Mérieux Inc, France). The bacterial strains were preserved in glycerine at −76°C for further use [1,2].

This experiment was approved by the guidelines of the Animal Use Protocol and the Institutional Animal Care and Use Committee (Protocol 97017) of the National Chiayi University.

2.2 Multiplex PCR identification

Bacterial DNA were purified using the Qiagen commercialized DNA extraction kit (DNeasy® Tissue Kit, New England Biolabs, Inc., USA). Four primer pairs used for species identification by a multiplex PCR are listed in Table 1 and included the pLG-1 and pLG-2 pair to amplify a 1,100-bp DNA fragment for for *L. garvieae* [10]; the Sdi-61 and Sdi-252 pair to amplify 192-bp PCR product for *S. agalactia* [10]; and the Strd-dyl and Dys-16S-23S-2 pair to amplify a 259-bp PCR product for *S. dysgalactiae* [22]; and . The LOX-1 and LOX-2 pair to amplify a 870-bp PCR product for S. *iniae* [10]. The 25-µl PCR reaction mixture contained 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.2 µM primers, and 0.5 U Taq DNA polymerase. The PCR conditions were as follows: predenaturation at 94 °C for 2 min; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 45 s; and a final extension at 72 °C for 5 min.

2.3 Pulsed field gel electrophoresis (PFGE) analysis

Genotyping of *L. garvieae* was determined by PFGE analysis with the methods previously [11]. Briefly, overnight bacteria were first embedded in 0.8 % agarose to make the plugs that were treated with lysozyme and then 1 mg/ml proteinase K at 50 °C. After washing with TE buffer, the plugs were digested with the restriction endonuclease ApaI. The macro-DNA fragments were separated by CHEF DRIII (BioRad, Taiwan) using a switching time of 4 s/70 s, 120°, and 6 V for 18 h for the first step and then a switching time of 4 s/70 s, 120°, and 4 V for 6 h. Different genotypes were determined by difference in banding pattern more than three bands. After electrophoresis, the gel was stained with 0.5 mg/mL EtBr for 30 min and the image was recorded under an ultraviolet light illumination. A camera system (Vilber Lourmat, EEC) was used to capture images, which were stored in TIFF format [23].

2.4 Antibiotic susceptibility testing

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Susceptibility to 13 antibiotics, including amoxicillin (AML), ampicillin (AMP), azithromycin (AZM), ceftriaxone (CRO), ciprofloxacin (CIP), clindamycin, (CLI), doxycycline (DOX), erythromycin (ERY), levofloxacin (LVX), moxifloxacin (MXF), oxytetracycline (TET), penicillin (PEN) and tetracycline (TET) were determined using the disk diffusion method[17,20]. The bacterial solution was adjusted to the same turbidity as the 0.5 McFarland standard and plated on blood agar at 37℃ for The antibiotic susceptibility was determined based on criteria of Clinical and Laboratory Standards Institute (CLSI) [24]. *Streptococcus pneumonia* ATCC49619 was used as the reference strain.

3. Results

3.1 Species identification

The multiplex PCR analysis identified *L. garvieae*, *S. agalactiae*, and *S. dysgalactiae*, but no *S. iniae* detected (Figure 1). The prevalence differed among microbial species with the highest for *Lactococcus garvieae* (146, 85.9%), followed by *Streptococcus dysgalactiae* (10, 5.9%) and *S. agalactiae* (9, 5.3%). Additionally, five double infections were obtained for *L. garvieae* and *S. agalactiae* in one case as well as *L. garvieae* and *S. dysgalactiae* in four cases (Table 2, Figure 1). These prevalence changed by yeas ranging from 78.3% to 90.9% for *L. garvieae*, 1.8% - 8.7% for *S. agalactiae*, and 1.6% - 9.7% for *S. dysgalactiae*.

3.2 Different prevalence among regions and microbial species

Most microbial were identified in Chiayi (108, 63.5%), followed by Yulin (44, 25.9%) and Tainan (18, 10.6%) (Table 2). The highest number of microbial in each counties was 43 in Chiayi and 10 in Tainan in 2014 and 21 in Yulin in 2016. *L. garvieae* and double species infection occurred in all three counties, while other two bacterial species was not found in Tainan. The prevalence of each microbial species differed among three counties, ranging from 72.9% in Yulin to 94.4% in Tainan for *L. garvieae*, from 3.7% in Chiayi to 11.4% in Yulin for *S. agalactiae*, and from 4.6% in Chiayi to 11.4% in Yulin for S. dysgalactiae (Table 3).,

3.3 PFGE genotyping of *L. garvieae* s

Totally 172 *L. garvieae* were investigated in this study. To differentiate the difference among these strains genetically, we used PFGE analysis and the restriction enzyme *Apa*I for genotyping. All DNA fragments were smaller than 194 kb, but all strains showed identical patterns (Figure 2), suggesting possibly that single genotype spread in these three counties. Furthermore, all strains lacked plasmid (dada not shown).

3.4 Antibiotic susceptibility analysis

Among 13 antibiotics, 146 strains from grey mullet were susceptible to AML, AZM, ERY, DOX, OXT and LVX, but non-susceptible rate differed depending on the antibiotics tested (Table 4). Almost all strains were resistance to CLI in 2013 – 2016 with reduced susceptibility to AMP (3, 2%), CRO (16, 11%) and PEN (30, 21%) of β -lactam group, CIP (74, 51%) and MXF (3, 2%) of fluoroquilonone group, and TET (36, 25%) of tetracycline group. The highest reduced susceptible rate was found in 2014 for CIP, CRO, MXF and TET, while the lowest reduced susceptible rate was found in 2016. Compared to the antibiotic susceptibility for the non-grey mullet, a decrease of resistant strains for CLI and an increase of resistance to AML, MXF, OXT and TET and the reduced susceptibility to AMP, CRO, and PEN were found for the non-grey mullet (Table 4). The antibiotic susceptibility of the strains from grey mullet differed among three counties with the higher non-susceptible rate was found for AMP, CIP, CRO, PEN in the strains isolated from chiayi

4. Discussion

4.1 Streptococcal infection associated with farmed fish species

The diseased fishes in this study exhibited corneas with a turbid, whitened, or red appearance and especially those with severe infection exhibited budging eyes on one or both sides. These symptoms were similar to the finding on rainbow trout infected with *L. garvieae* infection [25]. In 1997–2004, Lee *et al*. reported infection by Gram-positive streptococcus in 51 samples of cultured grey mullet in Taiwan [2]. Based on 16S rDNA sequence analysis, *L. garvieae* (45, 90%), *S. agalactiae* (3, 5%), and *S. dysgalactiae* (3, 5%), except *S. iniae,* Traditionally biochemical methods are used for bacterial identification. In this study we applied multiplex PCR to identify *L. garvieae*, *S. agalactiae*, *S. dysgalact iae*, and *S. iniae*, simultaneously [10]. We confirmed similar prevalence of the three bacterial species and no *S. iniae* from diseased grey mullet from 2013–2016 (Table 2). These results demonstrated no main change of the bacterial pathogens with the *L. garvieae* (87%) as the primary infectious species for grey mullet. In 265 tilapia infection from 2013–2015, *S. agalactiae* was the major streptococcal species, followed by *S. iniae*, *L. garvieae*, and *S. dysgalactiae* [8,11], indicating the infection of streptococcus species is associated with fish species, maybe due to host immunity and bacterial virulence factors. Previously, *L. garvieae* commercialized vaccine was applied for farmedctured amberjack and Japanese amberjack for protection, however, the fishes was die by *S. dysgalactiae* infection [26]. In this study, occasional double infection occurred mainly in 2014 and in the Yulin and Chiayi counties (Table 2 and 3). Therefore, multivalent Gram-positive *Streptococcus* vaccines should be developed in the future because of multiple species infection.

L. garvieae infection is referred to as lactococcosis that has been observed in aquatic animals worldwide [7,27,28]. In Taiwan, *L. garvieae* has been the primary bacterial parthogen to infect giant freshwater prawns, grey mullet, and trout [29,30,31].

Possibly the grey mullet *is more susceptible to L. garvieae* infection and resistant to *S. iniae* infection. However, the infection may be strain dependent due to differences in the virulence factors they carry. Therefore, PFGE method has been used to distinguish the genotypic differences between strains. The restriction enzyme *Apa*I digested PFGE analysis could separate 82 *L. garvieae* strains from Spain and Italy and different animals of fish, dairy cattle, or water buffalo into 19 pulsotypes [16]. Although restriction enzymes may affect PFGE DNA pattern, restriction enzymes *Apa*I and *Sma*I for studying *L. garvieae* genotypes showed identical pulsotype [23]. In our study, the presence of one pulsotype implies that the *L. garvieae* exists commonly in the aquatic farmed grey mullet in Taiwan [Figure 2].

4.2 Antibiotic susceptibility testing

Antibiotic susceptibility to 13 tested antibiotics differed between the strains from grey mullet and non-grey mullet, such that grey mullet strains were susceptible to 6 antibiotics, including AML, DOX, ERY, LEV, LIN, and OXT; while non-grey mullet strain only susceptible to AZM, ERY, and LEV and showed more reduced susceptible strains, except for antibiotics CLI and MXF (Table 4). These results indicate possible that the antibiotics are used differently for fish species. were found in This study conducted antibiotic susceptibility testing on the primary bacterium *L. garvieae* in grey mullet. Thirteen types of antibiotics disks were selected. Especially, the highest reduced susceptible strains was located in Chiayi county. Therefore regional antibiotic used shall be controlled more strictly. Antibiotic use can control clinical symptoms in sick fish, while the fishes that become the carrier for these bacterial species makes this disease difficult to eradicate [32].

L. garvieae can cause endocarditis in humans with symptoms of asitia, frailty, and dyspnea [33] and infect immunosuppressed patients with ,septicemia comorbid with liver abscess [34]. These findings may serve as references for public health studies on drug resistance. Among 13 antibiotics, AMP, AML, DOX, ERY and OXY are legally used on grey mullet in Taiwan, whereas AZM, CRO, CIP, CLI, LVX, MXF, PEN, and TET are inhibited. In this study, we observed the strains with the reduced susceptibility to CRO, CIP. CLI (100%), MXF, PEN, and TET. However, the number of the reduced susceptible strains decreased from 2014 to 2016 (Table 4). Our finding demonstrate the misuse of the antibiotics for human, not for aquatic fishes. The surveillances of human antibiotic shall be conducted for farmed fishes in assistane of public health.

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Author Contributions

Conceptualization, J.-H.W. and C.C. data analysis, C.-C.C and S.-Y C.; formal analysis, J.-L.L.; funding acquisition, C.-C.C. J.L.L. and J.-H.W. investigation, S.-Y. C. and J.-L.L; methodology, C.-C.C. and J.-H.W. supervision, J.-H.W. and C.C.; validation, C.C.; writing—original draft, J.-L.L. and J.-H.W.; final editing, C.C. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

Authors declare no conflict of interest.

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Legends

Figure 1. Electrophoresis of Multiplex PCR amplified PCR products. M: 100-bp size marker. The size of represented species is 1100 bp for *L. garvieae*, 259 bp for *S. dysgalactiae* and 192 bp for *S. agalactiae*. Lane 1: double infection of L. garvieae and *S. agalactiae*, Lane 2: double infection of L. garvieae and S. dysgalactiae, and L3: L. garvieae.

Figure 2. PFGE analysis of *Apa*I-digested chromosomal DNA fragment of *L. garvieae* . M: λ DNA size marker. Lane 1-13: different *L. garvieae* strains.

.

Figure 1.

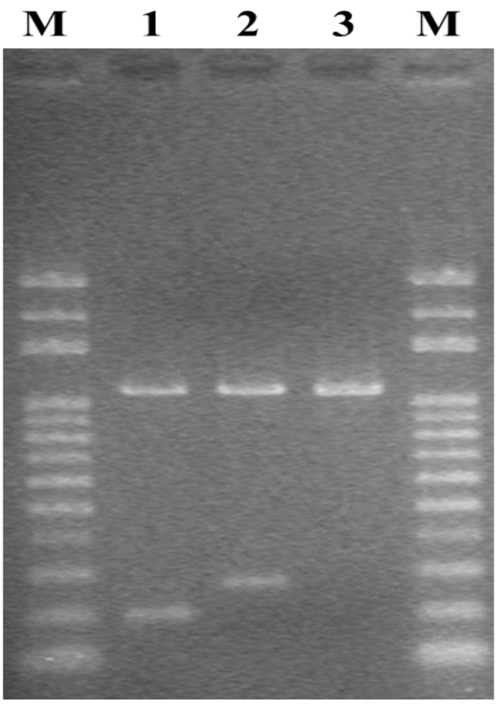

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Figure 2.

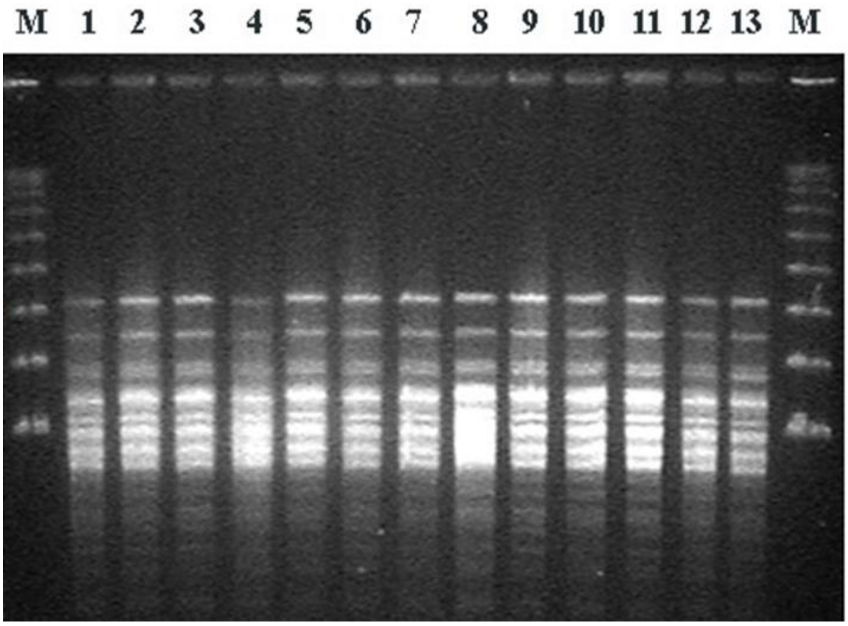

Table 1 . Multiplex PCR primers of pathogens used in this study.

Pathogens	Target gene (accession No.)	Primers	Sequences (5' to 3')	Product Size (bp)	Reference
Lactococcus garvieae	16S rDNA	pLG-1 pLG-2	CATAACAATGAGAATCGC GCACCTCGCGGGTTG	1100	[10]
Streptococcus agalactiae	16S-23S rDNA (U39765)	Sdi-61 Sdi-252	AGGAAACCTGCCATTTGCG CAATCTATTTCTAGATCGTGG	192	[10]
Streptococcus dysgalactiae	16S-23S rDNA	Strd-dyl Dys-16S-23S-2	TGGAACACGTTAGGGTCG CTTAACTAGAAAACTCTTGAT TATTC	259	[22]
Streptococcus iniae	LacO gene (Y07622)	LOX-1 LOX-2	AAGGGGAAATCGCAAGTGCC ATATCTGATTGGGCCGTCTAA	870	[10]

Table 2. Detection of Gram-positive streptococcus-like bacteria infection cases carried out by multiplex PCR between 2013-2016 in Taiwan.

Species/Region	2013	2014	2015	2016	Total
			Bacterial species		
L. garvieae	25 80.6%	53 86.9%	18 78.3%	50 90.9%	146 85.9%
S. agalactiae	2 6.5%	4 6.6%	2 8.7%	1 1.8%	9 5.3%
S. dysgalactiae	3 9.7%	1 1.6%	2 8.7%	4 7.3%	10 5.9%
Double infection	1 3.2%	3 4.9%	1 4.3%	0 0%	5 2.9%
			Region		
Yulin	11 25%	8 18.2%	4 9.1%	21 47.7%	44 25.9%
Chiayi	20 18.5%	43 39.8%	18 16.7%	27 25%	108 63.5%
Tainan	0 0%	10 55.6%	1 5.6%	7 38.9%	18 10.6%
Sum	31 18.2%	61 35.9%	23 13.5%	55 32.4%	170 100%

Table 3. Identification of Gram-positive streptococcus-like bacteria infection cases of different cities in Taiwan.

Species	Yulin	Chiayi	Tainan	Total
L. garvieae	32 72.7%	97 89.8%	17 94.4%	146 85.9%
S. agalactiae	5 (11.4%)	4 (3.7%)	0 0%	9 (5.3%)
S. dysgalactiae	5 (11.4%)	5 (4.6%)	0 0%	10 (5.9%)
Double infection	2 (4.5%)	2 (1.9%)	1 (5.6%)	5 (2.9%)

Table 4. Antibiotic susceptibility of 146 of *L. garvieae* strains from farmed grey mullet in 2013 - 2016.[1]

Year	Susceptibility[1]	AMP	AML	CLI	CIP	CRO	DOX	MXF	PEN	OXT	TET
Strains from grey mullet											
2013 n=25	R	1	0	24	0	1	0	0	0	0	0
	I		0	0	15		0			0	6
	S	24	25	1	10	24	25	25	25	25	19
2014 n=53	R	1	0	53	0	12	0	1	23	0	0
	I		0	0	38		0	2		0	22
	S	52	53	0	15	41	53	50	30	53	31
2015 n=18	R	1	0	18	0	3	0	0	6	0	0
	I		0	0	15		0			0	8
	S	17	18	0	3	15	18	18	12	18	10
2016 n=50	R	0	0	50	0	0	0	0	1	0	0
	I		0	0	6		0			0	0
	S	50	50	0	44	50	50	50	49	50	50
Total n=146	R	3 2%	0 0%	145 99%	0 0%	16 11%	0 0%	1 0.7%	30 21%	0 0%	0
	I		0 0%	0 0%	74 51%		0 0%	2 1.4%		0 0%	36 25%
	S	143 98%	50 100%	1 1%	72 49%	130 89%	50 100%	143 98%	116 79%	50 100%	110 75%
Strains from other fish species											
Non-grey Mullet n = 30	R	9 30%	7 23%	22 73%	0 0%	8 27%	0 0%	0 0%	13 43%	2 7%	2 7%
	I		0 0%	0 0%	9 30%		1 3%	0 0%		0	0
	S	21 70%	23 77%	8 27%	21 70%	22 73%	29 97%	30 100%	17 57%	28 (93%)	28 (93%)

[1]: R: resistant, I: intermediate, S: susceptible; AMP: ampicillin, AML: amoxicillin, CLI: clindamycin, CIP: ciprofloxacin, CRO: ceftriaxone, DOX: doxycycline, MXF: moxifloxacin, OXT: oxytetracycline, PEN: penicillin, and TET: tetracycline.

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Table 5. The antibiotic susceptibility of *L. garvieae* among three counties.[1]

Region	Susceptibility	AMP	CLI	CIP	CRO	MXF	PEN	TET
Yulin n=32	R	0 0%	32 100%	0	2 6%	0 0%	3 9%	0
	I		0 0%	13 1%		0 0%		3 9%
	S	32 100%	0 0%	19 59%	30 94%	100%	29 91%	29 91%
Chiayi n=97	R	3 3%	96 99%	0	12 12%	0 0%	22 23%	0
	I		0 0%	50 52%		1 1%		29 30%
	S	94 97%	1 1%	47 48%	85 88%	96 99%	75 77%	68 70%
Tainan n=17	R	0 0%	17 100%	0 0%	2 7%	1 7%	5 27%	0
	I		0 0%	9 90%		1 7%		4 20%
	S	12 100%	0 0%	8 40%	15 93%	15 87%	12 73%	13 80%

[1]: R: resistant, I: intermediate, S: susceptible; AMP: ampicillin, CLI: clindamycin, CIP: ciprofloxacin, CRO: ceftriaxone, MXF: moxifloxacin, PEN: penicillin, and TET: tetracycline.