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Carriage niches and Molecular Epidemiology of *Staphylococcus lugdunensis* and Methicillin-Resistant *S. lugdunensis* among Patients undergoing long-term renal replacement therapy

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Abstract

We collected nasal, axilla and groin swabs from 252 adult patients from two nephrology centers in Hong Kong. *Staphylococcus lugdunensis* carriage was detected in 51.6% patients(groin, 39.3%; axilla, 19.8% and nose, 17.9%). The carriage rates of methicillin-sensitive *S. lugdunensis* (MSSL) and methicillin-resistant *S. lugdunensis* (MRSL) were 46.0% and 8.3%, respectively. Independent risk factors for *S. lugdunensis* carriage included male sex (odds ratio [OR], 4.4), hemodialysis (OR 2.2) and aged 18-50 years (OR 2.4). The isolates belonged to ten pulsotype clusters (n=129) and eight singletons (n=8). All MRSL and most gentamicin and tetracycline resistant strains were found in a predominating sequence type 3 clone, designated HKU1, which accounted for 51.8% of all colonizing *S. lugdunensis* strains. The 21 MRSL isolates had SCC*mec* type V (*n*=18), type IV (*n*=2) and type I (*n*=1). The finding highlights the potential for dissemination of multidrug resistance through successful *S. lugdunensis* clones.

(Word counts 146)

1. Introduction

Coagulase-negative staphylococci (CoNS) comprise a large number of staphylococcal species that could be distinguished from *S. aureus* by the inability to clot plasma. Historically, only a few CoNS, including *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus* are identified to the species level while staphylococci would be reported by the generic name, CoNS. As conventional methods for bacterial identification lack the ability to discriminate all the staphylococcal species and these organisms are generally considered to be contaminants or to be avirulent, such a policy is widely adopted by clinical laboratories worldwide. In the laboratory, CoNS may be misidentified. Pinsky et al reported that biochemical identification was only 31% sensitive and 77% specific for *Staphylococcus lugdunensis* when tested against PCR-based method (Pinsky et al., 2009). With increasing access to molecular and mass-spectrometry techniques, an increasing number of CoNS recovered from blood cultures and other sterile body sites are being correctly identified to the species level (Chen et al., 2013; Ho et al., 2014). Complete genome sequencing and related analysis of *S. lugdunensis* has further improved our understanding of the potential pathogenicity determinants of the organism (Heilbronner et al., 2011; Tse et al., 2010). These developments have led to the recognition of *S. lugdunensis* as an unusually virulent CoNS but also open up knowledge gaps in the clinical epidemiology of this opportunistic pathogen (Frank et al., 2008). *S. lugdunensis* has become a well-documented cause of serious infections including

catheter-associated bacteremia/septicemia, native valve endocarditis, vascular aneurysms and osteomyelitis (Coates et al., 2014; Frank et al., 2008).

Notably, inguinal skin breaks (i.e. vascular access) have been associated with *S. lugdunensis* invasive infections including endocarditis which is frequently associated with embolization, valvular abscess and rapidly valve destruction (Lin et al., 2014; Frank et al., 2008). The unusual ability of *S. lugdunensis* to cause catheter-associated bacteremia/endocarditis and exit site infections highlights the potential for this organism to be a threat for renal patients on peritoneal dialysis and hemodialysis. One recent study found that 44.2% of healthcare-associated, invasive infections caused by *S. lugdunensis* involved patients who had end-stage renal disease that required dialysis (Lin et al., 2014). Therefore, this study was performed to investigate the prevalence and molecular epidemiology of *Staphylococcus lugdunensis* carriage in end-stage renal disease patients undergoing renal replacement therapy.

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2. Patients and methods

2.1 Study design and patients

This was a cross-sectional study. Swabs specimens were collected from patients who were treated in two nephrology centers within two regional hospitals (A and B) in Hong Kong during the period from November 2013 to Feburary 2014. The criteria for inclusions

were: (1) adults aged ≥18 years, (2) under any form of long-term renal replacement therapy including continuous ambulatory peritoneal dialysis, intermittent peritoneal dialysis and hemodialysis. Patients who were not capable of giving consent or deemed to be medically unstable (e.g. require immediate resuscitation or intensive care admission) by the in-charge doctor were excluded. A standardized form was used to obtain the following information from the medical records and through patient interviews: sex, age, ethnicity, type and duration of renal replacement, smoking habit, old age home residence, antibiotic use (current and within past four weeks), concurrent upper respiratory tract illness (URTI), and presence of other medical conditions including diabetes mellitus and chronic skin conditions (e.g. atopic dermatitis, psoriasis).

The study protocol was approved by the Institutional Review Boards of the University of Hong Kong and the Hospital Authority (reference numbers UW13-351 and KW/EX-13-138-69-15). Informed consents were obtained from the patients.

2.2 Microbiological methods

To assess *S. lugdunensis* carriage, three swabs (TRANSWAB; Medical Wire and Equipment Co. Ltd) were taken from each patient. One swab each was used to sample both sides of anterior nares, axillae and groins. All specimens were obtained by trained nurses. The swabs were transferred to the microbiology laboratory at the University of Hong Kong in

Amies transport media immediately. For selective isolation of *S. lugdunensis*, the swabs from each patient were inoculated into enrichment broths (each liter comprise 54.2g Giolitti-Cantoni broth, 10g D-trehalose dehydrate, 0.1g deferoxamine mesylate, 0.001g potassium tellurite and 0.025g phenol red) and incubated aerobically at 35 ^{o}C for 48 hours. Turbid broth were subcultured onto two culture plates (sheep blood and selective *S. lugdunensis* [SSL] agar) and incubated anaerobically for 48 hours (Ho et al., 2014). Colonies on the blood agar plates suggestive of *S. lugdunensis* (*Eikenella corrodens*-like odor, colony pleomorphism, and large β-hemolysis) and the SSL plates (gray colonies with purple halos) were picked for further investigations.(Ho et al., 2014) In spiking experiments, the protocol could detect <10 CFU of *S. lugdunensis* per swab in a mixed background of other staphylococci at 10^3 CFU per swab. Identification to the species level was achieved by biochemical tests and confirmation was obtained by PCR (Hirotaki et al., 2011; Ho et al., 2014). Antimicrobial susceptibility testing was performed by the disc diffusion method in accordance with the Clinical and Laboratory Standard Institute (CLSI) recommendations (cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, cotrimoxazole, erythromycin, fusidic acid, gentamicin, minocycline, tetracycline and rifampicin). Cefoxitin discs were used for phenotypic detection of methicillin resistance. The D-test was used to detect inducible resistance to clindamycin.(Ho et al., 2011) Quality control strains were included on each day of testing.

2.3 Molecular studies

Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to assess the genetic relatedness of the isolates. PFGE was performed by a rapid procedure (Ho et al., 2008). *Sma*I-restricted fragments were resolved in 1% SeaKem agarose (Bio-Rad Laboratories, Hercules, Calf.) at 6V/cm, with pulse times ramping from 5-15s for 16 hours following by 15 to 60s for 13 hours in a CHEF Mapper XA system. The banding patterns were analyzed with Gelcompar II software (Applied Maths). Dendrograms were created by means of the Dice coefficient and the UPMAG method. A similarity coefficient of 80% was selected to define pulsotype and designated as HKU1, 2, 3, etc. In each run, *S. lugdunensis* HKU09-01 (GenBnak accession CP001837) was included as internal control (Tse et al., 2010). MLST was performed on representative strains for each pulsotype with more than three isolates (Chassain et al., 2012) The SCC*mec* types were determined as described.(Ho et al., 2008; Ho et al., 2010). SCC*mec* types were designated according to the ccr type and *mec* class combinations (Ho et al., 2012b). A collection of strains with known SCC*mec* types were included as positive controls on each date of testing (Ho et al., 2008; Ho et al., 2010). Multiplex PCR assays were used for detection of genes encoding resistance to macrolides-linocosamindes-streptogramin B antibiotics (*ermA*, *ermB*, *ermC* and *mef*),

aminoglycosides (*aacA-aphD*), and tetracyclines (*tetK* and *tetM*) (Ho et al., 2011; Ho et al., 2012a).

2.4 Statistical analysis

The statistical package SPSS, version 17.0 (SPSS, Hong Kong), was used for all analyses. The chi-square test or the Fisher exact test was used for categorical variable. Continuous variables were tested by the Student *t* test. Univariate and multivariate analyses were used to assess risk factors for *S. lugdunensis* colonization. The following parameters were included in the multivariate analysis: age, sex, type of renal replacement therapy, duration of dialysis, current and past antibiotic use, smoking, old age home residence, diabetes mellitus, chronic skin condition and patient care hospital. The values of parameters are given as mean (± standard deviation [SD]) where appropriate. All reported *P* values were two-sided. A *P* value of less than 0.05 was considered to be statistically significant.

3. **Results**

3.1 Patient demographics and prevalence of S. lugdunensis carriage

A total of 252 patients, including 102 from hospital A and 150 from hospital B were enrolled. The type of renal replacement therapy was continuous ambulatory peritoneal dialysis in 125 (49.6%) patients, intermittent peritoneal dialysis in four (1.6%) patients and hemodialysis in

123 (48.8%) patients. The average duration on dialysis was 4.1 ± 5.0 years. The mean age of the patients was 61.9 ± 13.1 years. In this cohort, 98.0% were Chinese, 56.0% were male, 14.7% were old age home residents, 46.4% had diabetes mellitus (recent hemoglobin A1c level, 7.3 ± 1.6%), 7.9% had a chronic skin condition, 7.9% were current smokers, 8.7% had antibiotic use in the past four weeks, 6.3% were receiving antibiotic therapy and 0.4% had URTI at the time of study.

Overall, *S. lugdunensis* was detected in 194 swabs, of which 153 were detected by both blood agar and SSL plates, 9 were detected by blood agar plates only, and 32 were detected by SSL plates only (McNemar's test, $P<0.001$). The sensitivities of the blood agar and SSL plates were 83.5% (162/194) and 95.4% (185/194), respectively. *S. lugdunensis* carriage was detected in 130 (51.6%) patients (Table 1), of which 74 (29.4%) patients were positive in one niche, 48 (19.0%) patients in two niches and eight (3.2%) patients in three niches. The positive rate by carriage niche was highest in groin (39.3%) and similar rates were found for axilla (19.8%) and nose (17.9%) ($P < 0.001$ for groin vs. axilla and groin vs. nose). Among the 130 colonized patients, 109 patients had methicillin-sensitive *S. lugdunensis* (MSSL) alone, 14 patients had methicillin-resistant *S. lugdunensis* (MRSL) alone and 7 patients had both MSSL and MRSL. The carriage rates of MSSL and MRSL were 46.0% and 8.3%, respectively. Across all niches and for both MSSL and MSSL (Table 1), higher carriage rates were found in males than in females, and in hemodialysis patients than

in peritoneal dialysis patients. There were no significant difference in the carriage rates, niche of carriage and proportions of MRSL among patients enrolled from the two hospitals.

3.2 Potential risk factors for S. lugdunensis carriage

The risk factors for *S. lugdunensis* carriage were summarized in Table 2. *S. lugdunensis* carriage was significantly and positively associated with male sex, younger age and hemodialysis but not the other variables by univariate analyses. The age-stratified carriage rates were 63.6% in patients aged 18-50 years (young adults), 50.0% in patients aged 51-64 years (middle age adults), 45.6% in patients aged 65-74 years (seniors) and 49.0% in patients ≥75 years (elderly). Since smoking might only affect nasal carriage, this variable was further analyzed against carriage in the nose. Nonetheless, no significant association was found. The proportion of smokers among *S. lugdunensis* nasal carriers and nasal non-carriers was 6.8% (14/207) and 13.3% (6/45), respectively (P=0.139). In multivariate analysis, three variables were independently associated with *S. lugdunensis* carriage: male sex (odds ratio [OR], 4.4, 95% confidence interval [CI], 2.5-7.7, $P < 0.001$), hemodialysis (OR 2.2, 95% CI, 1.3-3.8, $P = 0.004$) and aged 18-50 years (OR 2.4, 95% CI, 1.2-4.8, $P = 0.012$). Due to the small number of MRSL carriers, the variables were not separately analyzed against MRSL carriage.

3.3 Antimicrobial susceptibility

In total, 137 unique isolates (116 MSSL and 21 MRSL isolates) from the 130 colonized patients were included in the susceptibility analysis. Overall, 54 isolates (39.4%) were nonsusceptible to at least one of the 11 antibiotics while 83 (60.6%) isolates were pan-susceptible. Drugs active against 98%-100% of both MSSL and MRSL isolates include chloramphenicol, ciprofloxacin, cotrimoxazole, minocycline, fusidic acid and rifampicin. Overall, the erythromycin resistance rate was 9.5% (13/137) including 8.6% (10/116) for MSSL and 14.3% (3/21) for MRSL. Among 13 erythromycin-resistant isolates, five and seven isolates had constitutive (cMLS phenotype) and inducible (iMLS phenotype) resistance to clindamycin, respectively. The remaining erythromycin-resistant isolate had the M phenotype (i.e. resistant to erythromycin only). Two antimicrobial agents were significant more likely to be nonsusceptible among MRSL isolates than MSSL isolates, including gentamicin (MRSL, 38.1% vs. MSSL, 13.8%, $P = 0.01$) and tetracycline (38.1% vs. 16.4%, $P = 0.02$). Three isolates (two MRSL and one MSSL) were multidrug-resistant (i.e. nonsusceptible to ≥3 non-β-lactam drugs).

3.4 Genotypic characteristics

The genotypic and epidemiologic features for the 137 (116 MSSL and 21 MRSL) isolates were summarized in Table 3. PFGE divided 129 (94.2%) isolates into ten pulsotypes

(i.e. clones) with at least three strains. The remaining eight isolates were singletons. A total of 17 isolates which were chosen to represent the pulsotypes were investigated by MLST. The ten PFGE clusters were found to belong to six STs (ST1, ST3, ST4, ST6, STnew1, STnew2) of which two STs were found for the first time in this study. The predominant clone, HKU1 accounted for 57.7% (79/137) of all strains. Antimicrobial resistance varied by clones. Strikingly, HKU1 accounted for 100% (21/21) of the methicillin-resistant isolates, 91.7% (22/24) of the gentamicin-resistant isolates, 92.6% (25/27) of the tetracycline-resistant isolates and 46.2% (6/13) of the erythromycin-resistant isolates. In contrast, HKU1 isolates only accounted for 32.5% (27/83) of the pan-susceptible isolates. The rate of pan-susceptibility among HKU1 and non-HKU1 isolates were 38.0% (27/71) and 84.8% (56/66), respectively (P<0.001). The 21 MRSL isolates had either SCC*mec* type V (n=18, seven from hospital A and 11 from hospital B), type IV (n=2, hospital A only) and type I (n=1, hospital B only). All three multidrug-resistant strains (two MRSL/SCCmecV from hospital A and one MSSL from hospital B) belonged to HKU1 clone. PCR revealed that 92.3% (12/13) erythromycin-resistant isolates had *ermA* (n = 1) or *ermC* (n = 11) genes; 100% (24/24) gentamicin-resistant isolates had *aacA-aphD* gene and 100% (27/27) tetracycline-resistant isolates had the *tetK* gene (Table 3).

4. **Discussion**

In this study, exploring three niches, *S. lugdunensis* carriage was found to be prevalent among patients undergoing long-term renal dialysis, especially in the groins. The carriage rates for axilla and groin are comparable to those previously reported, while the carriage rate for the nose is much higher. Reported carriage rates of *S. lugdunensis* in healthy adults and patients ranged 4.7-9.3% for nose, 20-22% for axilla and 22.1-32% for groin (Bieber and Kahlmeter, 2010; Koziol-Montewka et al., 2006; Mee-Marquet et al., 2003; Ohara-Nemoto et al., 2008). In our hemodialysis patients, the *S. lugdunensis* carriage rate in the nose (22.0%) is 4.7 folds higher than the rate (4.7%) reported in a previous study involving 43 hemodialysis patients (Koziol-Montewka et al., 2006). This is likely due to the better ability of our culture approach in recovering *S. lugdunensis*, especially in niche carrying small number of the organism. All previous carriage studies employed blood agar plates for recovery of *S. lugdunensis* without enrichment (Bieber and Kahlmeter, 2010; Koziol-Montewka et al., 2006; Mee-Marquet et al., 2003; You et al., 1999). while this study included an enrichment step and used a novel selective agar medium (Ho et al., 2014). As recently reported by our group (Ho et al., 2014), when wound specimens were directly plated onto SSL plates, the medium had a sensitivity of 94.4% as compared with 19.4% for blood agar plates (Ho et al., 2014). This compared with the 95.4% and 83.5% sensitivities for SSL plates and blood agar plates, respectively, in the present study.

This study found that younger age, male sex and hemodialysis were independently associated with carriage of *S. lugdunensis*. The findings are novel as previous studies have not been able to detect risk factor for carriage because of small sample size or study design (Bieber and Kahlmeter, 2010; Koziol-Montewka et al., 2006; Mee-Marquet et al., 2003; Ohara-Nemoto et al., 2008). The three risk factors were identified out of a list of variables that are recognized to increase carriage of *S aureus*, which is closely related to *S. lugdunensis* (Weidenmaier et al., 2012; Johannessen et al., 2012). The increased rate of *S. lugdunensis* carriage among patients with those characteristics may indicate sex-, age- or disease-related biological differences (e.g. hormonal effect, bacterial-host cell interaction), more frequent healthcare contacts, repeated breaches of their skin from vascular access, personal hygiene or habit of nose-picking or scratching (Weidenmaier et al., 2012; Johannessen et al., 2012). Since heme promotes the growth of *S. lugdunensis*, one could postulate that the use of supplementary iron in hemodialysis may also facilitate carriage (Brozyna et al., 2014). Interestingly, several established risk factors for *S. aureus* carriage including diabetes mellitus, chronic skin disorders, were found not to be associated with *S. lugdunensis* (Tong et al., 2012). Therefore, *S. lugdunensis* and *S. aureus* might utilize different mechanisms to colonize the skin and nose (Coates et al., 2014).

This study showed that certain resistance phenotypes in association with SCC*mec* type V, *ermC* and *tetK* are emerging among strains from patients with end-stage renal failure.

Strikingly, all methicillin resistance and a majority of the gentamicin and tetracycline resistance were found in a predominating clone, ST3/HKU1, which accounted for 51.8% of all colonizing *S. lugdunensis* strains. In contrast, colonizing and infecting strains from previous studies were generally susceptible to multiple antibiotics (Frank et al., 2008). Currently, methicillin resistance remains rare and MRSL strains reported to cause infections, as for colonizing strains in the present study were healthcare-associated (Lin et al., 2014; Liu et al., 2012). It is postulated that the expansion of this HKU1 clone may due to its greater ability to cause persistent colonization, acquire resistance determinants, and nosocomial transmission. Previous studies have found ST3 to be involved in skin and soft tissue, osteoarticular and material device-associated infections recovered from different geographical areas (Chassain et al., 2012).

It should be pointed out that *S. lugdunensis* has a clonal population structure and the pathogen evolves by mutation instead of homologous recombination. Therefore, the discriminatory power of PFGE and MLST typing for epidemiological studies of this organism would be lower than that for *S. aureus* (Chassain et al., 2012). In the future, it should be informative to use genome sequencing techniques to delineate the microevolution of this pathogen and to find out whether the resistant strains within HKU1 represent a unique subclone (Heilbronner et al., 2011; Tse et al., 2010). As the present study is cross-sectional in nature, no inference may be made about the carriage dynamics. Longitudinal studies are

required to define the distribution of *S. lugdunensis* noncarriers, intermittent carriers and persistent carriers, as well as the risk of developing infection following carriage.

In conclusion, by using a novel culture method, this study provides useful information on risk factors for *S. lugdunensis* carriage and the emerging resistance phenotypes in this pathogen among patients with end-stage renal disease undergoing dialysis therapy. Our finding highlights the potential for dissemination of multidrug resistance through successful *S. lugdunensis* clones. Thus, continued efforts to enhance hygiene in dialysis centers and among high risk patients is necessary to slow down the further emergence of this pathogen.

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Table 1

Patients with colonization

	No (%) with *S. lugdunensis*				
	Female (n=111)	Male (n=141)	Peritoneal dialysis (n=129)	Hemodialysis (n=123)	Total (n=252)
Niche					
Nose	9 (8.1)	36 (25.5)	18 (14.0)	27 (22.0)	45 (17.9)
Axilla	17 (15.3)	33 (23.4)	20 (15.5)	30 (24.4)	50 (19.8)
Groin	23 (20.7)	76 (53.9)	42 (32.6)	57 (46.3)	99 (39.3)
Any	37 (33.3)	93 (66.0)	56 (43.4)	74 (60.2)	130 (51.6)
≥2	9 (8.1)	47 (33.3)	21 (16.2)	35 (28.4)	56 (22.2)
Phenotype					
MSSL	35 (31.5)	81 (57.4)	50 (38.8)	66 (53.7)	116 (46.0)
MRSL	2 (1.8)	19 (13.5)	8 (6.2)	13 (9.8)	21 (8.3)

MSSL, methicillin-sensitive *S. lugdunensis*; MRSL, methicillin-resistant *S. lugdunensis*

Table 2

Univariate analysis of potential risk factors for *S. lugdunensis* carriage

Factor	*S. lugdunensis* carriage		*P*
	No (*n* = 122)	Yes (*n* = 130)	
Male, %	39.3	71.5	<0.001
Age, mean ±SD, y	63.6 ± 12.4	60.3 ± 13.6	0.045
Hemodialysis, %	40.2	56.9	0.008
Duration of dialysis, mean ±SD, y	4.2 ± 4.9	4.0 ± 5.1	0.795
Current antibiotic use, %	5.7	6.9	0.700
Prior antibiotic use, %	9.8	7.7	0.547
Current smoker, %[a]	4.9	10.8	0.086
Old age home resident, %	15.6	13.8	0.699
Diabetes mellitus, %	45.9	46.9	0.871
Chronic skin disease, %	6.6	9.2	0.433
Medical care in hospital A, %	39.3	41.4	0.723

MRSL, methicillin-resistant *S. lugdunensis*; OR, Odds ratio; SD, standard deviation; URTI, upper respiratory tract illness; y, years.

Table 3

Genotypic and phenotypic characteristics of 116 methicillin-sensitive and 21 methicillin-resistant *S. lugdunensis* isolates

Pulsotype	n	MLST (allelic profile)	No. by hospital		No. with phenotypes[c]				Resistance determinants[c]
			A	B	Mth-R	Ery-R	Gen-R	Tet-R	
HKU1	71	ST3 (4-3-4-2-3-4-6)	34	37	21[b]	6	22	25	*aacA-aphD* (22), *ermC* (6), *tetK* (25)
HKU2	18	ST6 (1-1-1-1-1-1-2)	5	13	0	2	0	1	*ermC* (2), *tetK* (1)
HKU3	9	ST21 (7-3-1-1-1-7-5)[a]	4	5	0	0	1	0	*aacA-aphD* (1)
HKU4	9	ST4 (6-4-3-1-1-3-3)	5	4	0	0	0	0	None
HKU5	5	ST4 (6-4-3-1-1-3-3)	3	2	0	0	0	0	None
HKU6	5	ST6 (1-1-1-1-1-1-2)	2	3	0	1	0	0	*ermC* (1)
HKU7	3	ST22 (8-3-8-1-1-3-5)[a]	1	2	0	0	1	0	*aacA-aphD* (1)
HKU8	3	ST4 (6-4-3-1-1-3-3)	0	3	0	0	0	0	None
HKU9	3	ST6 (1-1-1-1-1-1-2)	1	2	0	1	0	0	*ermC* (1)
HKU10	3	ST1 (1-1-1-1-1-1-1)	0	3	0	0	0	0	None
Singleton	8	Not determined	4	4	0	3	0	1	*ermA* (1), *ermC* (2), *tetK*(1)
Total	137		59	78	21	13	24	27	

Ery-R, erythromycin-resistant; Gen-R, gentamicin-resistant; Tet-R, tetracycline-resistant; Mth-R, methicillin-resistant; MLST, multilocus sequence typing; ST, sequence type.

[a] New STs found for the first time in this study.

[b] Three, eight and eight of them had co-resistance to erythromycin, gentamicin and tetracycline, respectively.

[c] *aacA-aphD*, aminoglycoside resistance gene encoding the bifunctional enzyme, 6'-aminoglycoside N-acetyltransferase/2"-aminoglycoside phosphotransferase; *ermB and ermC*, genes encoding macrolide-lincosamide-streptogramin B resistance. Parentheses indicate the number of isolates with the resistance determinant.

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