Distribution and phenotypic and genotypic detection of a metallo- β -lactamase, CphA, among bacteraemic *Aeromonas* isolates

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The objectives of the study were to investigate the distribution of *cphA*-related genes (*cphA*) encoding a CphA metallo- β -lactamase (MBL) among 51 consecutive *Aeromonas* blood isolates and to compare different phenotypic methods for detecting CphA. The presence of *cphA* was detected by PCR. Four phenotypic methods, the imipenem-EDTA combined disc test, imipenem-EDTA MBL Etest, agar dilution test and modified Hodge test (MHT), were used to detect imipenem susceptibility and MBL production. The results showed that 35 (69%) blood isolates had *cphA*. All (100%) of 16 *Aeromonas aquariorum* isolates and 12 *Aeromonas veronii* isolates, and 4 (80%) of 5 *Aeromonas hydrophila* isolates, carried *cphA*, but none of 15 *Aeromonas caviae* isolates did. With the standard inocula, irrespective of the presence or absence of *cphA*, all but one (50, 98%) isolates were susceptible to imipenem tested by disc diffusion, Etest and agar dilution (10^4 c.f.u. spot inocula), and did not exhibit MBL production by the imipenem-EDTA combined disc test and MBL Etest. By the agar dilution test using large inocula (10^7 c.f.u.), 34 (97%) of 35 *cphA*⁺ isolates had imipenem MICs of $\geq 16 \mu\text{g ml}^{-1}$, higher than the susceptible breakpoint ($4 \mu\text{g ml}^{-1}$), and demonstrated positive results for the MHT, while one *cphA*⁺ and all 17 *cphA*⁻ isolates had imipenem MICs of $\leq 4 \mu\text{g ml}^{-1}$. In conclusion, the distribution of *cphA* among aeromonads is species-specific, found in *A. aquariorum*, *A. veronii* and *A. hydrophila*, and the MHT may be a phenotypic screening test for CphA production.

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Abbreviations: CLSI, Clinical and Laboratory Standards Institute; MBL, metallo- β -lactamase; MHT, modified Hodge test.

The GenBank/EMBL/DDBJ accession numbers for the partial sequences of the *rpoB* genes of *Aeromonas aquariorum* MDC47^T (=BCRC 17946^T) and *A. aquariorum* A2-13, A2-42, A2-56, A2-61, A2-70, A2-94, A2-98, A2-107, A2-155, A2-157 and A2-159 are JF972604–JF972615, and those for the partial sequences of the *cphA*-related genes of *A. aquariorum* MDC47^T (=BCRC 17946^T) and *A. aquariorum* A2-13, A2-42, A2-56, A2-61, A2-70, A2-94, A2-98, A2-107, A2-157 and A2-159 are JF972618–JF972628.

A supplementary figure is available with the online version of this paper.

INTRODUCTION

Aeromonads, oxidase-producing Gram-negative rods, are aquatic micro-organisms and have been implicated in a variety of human diseases, ranging from mild gastroenteritis (Janda & Abbott, 2010) to fatal septicaemia, peritonitis/abdominal sepsis (Wu *et al.*, 2009) and necrotizing fasciitis (Lee *et al.*, 2008). Three principal classes of chromosomally mediated β -lactamases have been recognized in *Aeromonas* species, i.e. Ambler class C cephalosporinases, class D penicillinases and class B

metallo- β -lactamases (MBLs) (Janda & Abbott, 2010). Fosse *et al.* (2003) characterized the strains producing these β -lactamases into five major phenotypes: *Aeromonas hydrophila* complex/class B, C and D β -lactamases; *Aeromonas caviae* complex/class C and D β -lactamases; *Aeromonas veronii* complex/class B and D β -lactamases; *Aeromonas schubertii* spp./class D β -lactamase; and *Aeromonas trota* spp./class C β -lactamase.

The most commonly found MBL in *Aeromonas* species is CphA, which was first named in 1991 (Massidda *et al.*, 1991). In addition to CphA, other MBLs have been identified in aeromonads, including ImiS (Walsh *et al.*, 1998), IMP-19 (Neuwirth *et al.*, 2007) and VIM (Libisch *et al.*, 2008). CphA, a metalloenzyme, is inhibited by metal chelators, such as EDTA (Massidda *et al.*, 1991; Segatore *et al.*, 1993). Compared with other class B enzymes, CphA MBL has a very specific substrate profile, being active on penem and carbapenem only, and not on penicillin and cephalosporins (Segatore *et al.*, 1993). The distribution of *cphA*-related genes is species-related: they are mainly found in isolates of *A. hydrophila*, *A. veronii* and *Aeromonas jandaei*, but not in *A. caviae* (Balsalobre *et al.*, 2009; Rossolini *et al.*, 1995). However, CphA MBL production is not easily detected by *in vitro* susceptibility testing unless using large inocula. Tested by the agar dilution method with a larger inoculum (10^8 c.f.u.), the imipenem MICs for *Aeromonas* strains with *cphA*-related genes were ≥ 4 mg ml $^{-1}$, higher than the susceptible breakpoint, in a study by Rossolini *et al.* (1995).

Recent developments in the field of *Aeromonas* taxonomy based on genetic identification have led to a reclassification of aeromonads and to the description of novel species. For example, several clinical isolates from Taiwan were identified as *A. aquariorum* (Figueras *et al.*, 2009), a recently described species found in aquarium water and ornamental fish (Martínez-Murcia *et al.*, 2008). Furthermore, *A. aquariorum* was found to be widely distributed in clinical and environmental specimens, and could be misidentified as *A. hydrophila* (Aravena-Román *et al.*, 2011). *A. aquariorum* can be associated with diarrhoea, bacteraemia, skin and soft tissue infections, and other extraintestinal infections, and clinicians should be aware of this novel clinically relevant species (Figueras *et al.*, 2009). An understanding of the β -lactamase types intrinsically harboured in this novel species would be a guide for antimicrobial therapy. However, current knowledge about *cphA* distribution in this novel species is limited.

In this work, we investigated the prevalence of *cphA*-related genes and evaluated different phenotypic methods for detecting CphA MBL in reference strains and blood isolates of different *Aeromonas* species, trying to expand the knowledge of the occurrence and distribution of CphA MBL and *cphA*-related genes in the members of the genus *Aeromonas*, with special attention to the novel species *A. aquariorum*.

METHODS

Bacterial isolates and species identification. Consecutive *Aeromonas* blood isolates at National Cheng Kung University Hospital, a medical centre in southern Taiwan, were collected from June 1999 to June 2001 and stored at -70°C until use. Only the first isolate of the same species from each patient was studied. During the study period, 51 blood isolates from 47 patients were available. An *Aeromonas* blood isolate, A2-1000804, which was collected in 2011 from a patient with a febrile episode while receiving the 7th day of ertapenem therapy for biliary tract infection caused by *Escherichia coli* producing extended-spectrum β -lactamase, was also included. The genus *Aeromonas* was identified by positive oxidase test, fermentation of D-glucose, motility, the absence of growth in 6.5% sodium chloride and resistance to the vibriostatic agent O/129 (150 μg), and was confirmed by the GNI Plus system (Vitek Systems; bioMérieux) or API 20E system (bioMérieux). Species identification was based on both the Vitek system or API 20E system and sequence analyses of the partial *rpoB* gene obtained by PCR with the primers PasrpoB-L (5'-GCAGTGAAAGARTTCTTGGTTC-3') and RpoB-R (5'-GTTGC-ATGTTNGNACCCAT-3') (Küpfer *et al.*, 2006). The sequences of amplified DNA products, around 500 bp, were compared with reference sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). Based on the *rpoB* results, isolates with a dissimilarity value $\leq 0.9\%$ for a given type strain were identified to that species (Lamy *et al.*, 2010).

A. aquariorum MDC47^T (=BCRC 17946^T), *A. veronii* bv. sobria ATCC 9071^T, *A. hydrophila* ATCC 7966^T and *A. caviae* ATCC 13136^T were used as the reference strains for different *Aeromonas* species.

Detection of *cphA*-related genes. The presence of *cphA*-related genes was detected by both PCR amplification and DNA sequence analyses and the colony hybridization technique. PCR was performed with primers designed according to the *A. hydrophila* AE036 *cphA* gene (GenBank accession no. X57102), i.e. ANY-SSD/F (F: 5'-GCTTAGAGCTCCTAAGGAGCAAGATGAAAGGTTGG-3'; R: 5'-GCATAGGTACCTTATGACTGGGGTGCGCCTTG-3') (Massidda *et al.*, 1991; Talavera *et al.*, 2006), with similar conditions for the *rpoB* gene amplification except for the annealing temperature, which was changed to 53°C . The procedures for colony hybridization followed the manufacturer's instructions (DIG DNA Labelling kit; Roche), in which the hybridization probes were designed from the amplified DNA products of isolates of *A. aquariorum* A2-5, *A. veronii* A2-7 and *A. hydrophila* A2-11, around 720 bp. The amplified DNA sequences of these three isolates were 94% identical to those of the *A. hydrophila* AE036 *cphA* genes.

All imipenem-non-susceptible isolates were examined for other genes encoding MBLs, including *bla*_{IMP} and *bla*_{VIM} genes by previously described PCR primers for IMP and VIM families and conditions (Balsalobre *et al.*, 2009). Two isolates of *Pseudomonas aeruginosa* with the *bla*_{VIM-3} gene and two isolates of *Klebsiella pneumoniae* with the *bla*_{IMP-8} gene were used as positive control strains.

Imipenem susceptibility and expression of MBL. The discs with imipenem (10 μg) alone or associated with EDTA (10 μl , 0.5 M), the MBL Etest (BD BIODISK, imipenem 4–256 $\mu\text{g ml}^{-1}$ and imipenem 1–64 $\mu\text{g ml}^{-1}$ + EDTA) and the agar dilution test (imipenem 0.5–16 $\mu\text{g ml}^{-1}$) were used to detect imipenem susceptibility and MBL production. The procedures for the disc diffusion test, Etest and agar dilution test followed the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009, 2010). As recommended by the CLSI, standard inocula of 0.5 McFarland for the disc diffusion test and Etest and spot inocula of 10^4 c.f.u. for the agar dilution test were used. Larger inocula of 1.5 McFarland for the disc diffusion test and Etest and spot inocula of 10^7 c.f.u. for the agar dilution test were also adopted. Interpretation of imipenem susceptibility followed the CLSI

recommendations for *Aeromonas* species (CLSI, 2010), and results determined with both standard and large inocula adopted the same interpretative criteria for comparison in this study. Isolates that did not fit the criteria of 'susceptible', and belonged to the categories of 'intermediate' and 'resistant', were classified as being 'non-susceptible'. A >7 mm increase in the inhibition zone diameter in the presence of imipenem associated with EDTA in comparison with that of imipenem alone was interpreted as evidence of MBL production (Yong *et al.*, 2002). By MBL Etest, the expression of MBL was reflected by a reduction of imipenem MIC by at least eightfold, the appearance of a phantom zone or deformation of the imipenem ellipse in the presence of EDTA. *P. aeruginosa* ATCC 27853^T, negative for MBL, was used as the quality control strain. The modified Hodge test (MHT) followed the CLSI standards of a confirmatory test for carbapenemase production in members of the *Enterobacteriaceae* (CLSI, 2009), and an ertapenem disc (10 µg) was the carbapenem used.

Clinical data. Medical records of patients with bacteraemia due to *Aeromonas* species during the study period were reviewed. The clinical outcome of patients treated by carbapenems was analysed.

RESULTS

Species and *cphA*-related genes distribution

By sequence analyses of the partial *rpoB* genes, 16, 11 and 8 isolates were identified as *A. aquariorum*, *A. caviae* and *A. veronii*, with a dissimilarity value $\leq 0.9\%$ for the *rpoB* genes of *A. aquariorum* DSM 18362^T (GenBank accession no. FM210471), *A. caviae* strain V83 (GenBank accession no. AY851107) and *A. veronii* strain JF2638 (GenBank accession no. AY851124), respectively. Based on typical phenotypic reactions in the Vitek or API 20E system and *rpoB* gene sequences with 98.6–98.8% identity to that of *A. caviae* strain V83, another four isolates were identified as *A. caviae*. Likewise, four isolates were identified as *A. veronii* because their *rpoB* gene sequences had 98.6–98.8% identity to that of *A. veronii* strain JF2638 and they showed typical phenotypic reactions, and five isolates were identified as *A. hydrophila* because their *rpoB* sequences had 98.0–98.6% identity to that of *A. hydrophila* ATCC 7966 (GenBank accession no. CP000462). However, species identification of three isolates was not possible due to incompatible results of the phenotypic identification and the *rpoB* sequencing. Overall, 51 blood isolates were identified as *A. aquariorum* (16, 31%), *A. caviae* (15, 29%), *A. veronii* (12, 24%), *A. hydrophila* (5, 10%) and *Aeromonas* species (3, 4%). Some of the isolates had been described previously (Wu *et al.*, 2007; Figueras *et al.*, 2009). The GenBank accession numbers for the *rpoB* gene sequences of *A. aquariorum* MDC47^T and 11 published *A. aquariorum* blood isolates are JF972604–JF972615. The A2-1000804 isolate with 99.2% sequence identity of the *rpoB* gene to that of *A. aquariorum* DSM 18362^T was determined as *A. aquariorum*.

By PCR amplification and sequence analysis, *A. aquariorum* MDC47^T, *A. aquariorum* A2-1000804, *A. veronii* bv. *sobria* ATCC 9071^T, *A. hydrophila* ATCC 7966^T and 35 (69%) of the 51 blood isolates had *cphA*-related genes. For

the 51 blood isolates, the results of colony hybridization were concordant with those of PCR, except for *Aeromonas* species A2-84, which was hybridization-negative but PCR-positive. Combining the results of PCR and colony hybridization, all *A. aquariorum* (16/16, 100%) and *A. veronii* (12/12, 100%) isolates carried *cphA*, while none of 15 *A. caviae* isolates did. Four (80%) of five *A. hydrophila* isolates and three *Aeromonas* species had *cphA*.

With the exception of *A. aquariorum* A2-155, the DNA sequences of PCR products of *A. aquariorum* MDC47^T and A2-1000804, *A. veronii* bv. *sobria* ATCC 9071^T, *A. hydrophila* ATCC 7966^T and 34 blood isolates, around 720 bp, corresponded to that of the *A. hydrophila* AE036 *cphA* gene (GenBank accession no. X57102) with an identity of 93–95%. The PCR product of A2-155 had an extra 219 nt inserted sequence compared to *A. hydrophila* AE036 *cphA* genes (Fig. S1). The GenBank accession numbers for the sequences of *cphA*-related genes of *A. aquariorum* MDC47^T and 10 published *A. aquariorum* blood isolates (with the exception of A2-155) are JF972618–JF972628.

cphA and imipenem susceptibility

The results of three phenotypic methods for imipenem susceptibility testing of reference strains and 51 blood isolates are shown in Tables 1, 2 and 3, respectively. Using the three methods of disc diffusion, Etest and agar dilution to study imipenem susceptibility among 35 isolates carrying *cphA*, imipenem non-susceptibility was detected in one (3%) isolate with the standard inoculum, while it was detected among 12 (34%), 17 (49%) and 34 (97%) isolates, respectively, with a large inoculum. Of note, 34 *cphA*⁺ isolates were resistant to imipenem (MICs ≥ 16 µg ml⁻¹), as studied by the agar dilution test with a large inoculum, and were negative for *bla*_{IMP} and *bla*_{VIM} genes. By either the imipenem-EDTA combined disc test or MBL Etest, only 1 (3%) of 34 *cphA*⁺ isolates exhibited an MBL-producing phenotype, while 10 (29%) and 17 (49%) isolates, respectively, did so with a large inoculum. None of 16 *cphA*⁻ isolates exhibited imipenem non-susceptibility or an MBL-producing phenotype with either a standard or large inoculum. Similarly, imipenem non-susceptibility in three *cphA*⁺ reference strains was only demonstrated by the agar dilution test with a large inoculum.

Unlike most *cphA*⁺ strains, two *cphA*⁺ *A. aquariorum* isolates, A2-107 and A2-1000804, constantly exhibited imipenem non-susceptibility (MIC: 24 and >32 µg ml⁻¹ by Etest with a standard inoculum, respectively) and expressed the MBL-producing phenotype by the imipenem-EDTA combined disc test and MBL Etest with either a standard or large inoculum. In contrast, one *cphA*⁺ *A. aquariorum* isolate, A2-155, was always susceptible to imipenem by the three susceptibility tests with either inoculum.

Table 1. Presence of *cphA*-related genes (*cphA*), *in vitro* imipenem (IP) susceptibility studied with standard and high inocula, and the MHT in *Aeromonas* and *Pseudomonas* reference strains

Reference strain	<i>cphA</i> *	Disc diffusion, zone diameters† (mm)		Etest MIC‡ (µg ml ⁻¹)		Agar dilution MIC† (µg ml ⁻¹)		MHT
		IP/IP-EDTA (Δ) (0.5 McFarland)	IP/IP-EDTA (Δ) (1.5 McFarland)	IP/IP-EDTA (0.5 McFarland)	IP/IP-EDTA (1.5 McFarland)	IP (10 ⁴ c.f.u.)	IP (10 ⁷ c.f.u.)	
<i>A. aquariorum</i> MDC47 ^T	+	25/26 (1)	21/21 (0)	<4/<1	<4/<1	<1	>16	+
<i>A. veronii</i> bv. <i>sobria</i> ATCC 9071 ^T	+	25/26 (1)	21/23 (2)	<4/<1	<4/<1	<1	8	+
<i>A. hydrophila</i> ATCC 7966 ^T	+	23/24 (1)	22/24 (2)	<4/<1	<4/<1	<1	>16	+
<i>A. caviae</i> ATCC 13136 ^T	-	36/36 (0)	32/33 (1)	<4/<1	<4/<1	<1	<1	-
<i>P. aeruginosa</i> ATCC 27853 ^T	-	25/26 (1)	24/24 (0)	<4/<1	<4/1	2	4	-

*Detected by PCR.

†Imipenem non-susceptibility: an inhibition zone diameter <16 mm by disc diffusion test or an MIC >4 µg ml⁻¹ by Etest or agar dilution method.

MHT and imipenem susceptibility

By the MHT, carbapenemase activity was detected among *A. aquariorum* MDC47^T and A2-1000804, *A. veronii* bv. *sobria* ATCC 9071^T, *A. hydrophila* ATCC 7966^T and 34 (97 %) of 35 *cphA*⁺ blood isolates, and was absent in *A. caviae* ATCC 13136^T and 14 (88 %) of 16 *cphA*⁻ blood isolates (Fig. 1a–e). The MHT results for one *cphA*⁺ isolate (*A. aquariorum* A2-155) (Fig. 1f) and two *cphA*⁻ *A. caviae* isolates were indeterminate because the indentation between the aeromonad streak and the margin of ertapenem inhibition zone was not obvious. The indeterminate MHT result for A2-155 was correlated with that of the agar dilution test with a large inoculum, in which imipenem non-susceptibility could not be demonstrated either. Overall, the MHT could detect carbapenemase activity in 34 (100 %) imipenem-nonsusceptible blood isolates.

Clinical data

Among 47 patients with *Aeromonas* bacteraemia from June 1999 to June 2001, only one patient had ever received carbapenem therapy. This patient with *cphA*⁺ *A. veronii* and *Enterobacter cloacae* bacteraemia received an aminoglycoside active against both *E. cloacae* and *A. veronii* at bacteraemia onset and imipenem 1 week after bacteraemia onset and survived. The clinical condition of the patient with breakthrough bacteraemia due to *A. aquariorum* A2-1000804 during ertapenem therapy improved with ciprofloxacin therapy.

DISCUSSION

The conserved sequence of the *rpoB* gene, encoding the β-subunit of the DNA-dependent RNA polymerase in the genus *Aeromonas*, has been used for delineating *Aeromonas* species (Küpfer *et al.*, 2006; Lamy *et al.*, 2010). Therefore, in the present study, the *Aeromonas* species were identified by, in addition to traditional biochemical reactions, sequence analyses of the partial *rpoB* genes. We found that *A. aquariorum* was a predominant species causing bacteraemia. Such a fact highlights the potential clinical impact of *A. aquariorum* in, at least, southern Taiwan. *A. aquariorum* has been reported to be resistant to amoxicillin, cephalothin and cefoxitin (Figueras *et al.*, 2009), but the corresponding types of β-lactamases have not been studied. Therefore, information on the β-lactamase profile of this clinically important species is valuable for rational recommendations for antimicrobial therapy and justifies the clinical relevance of the present study.

By PCR and the colony hybridization technique, the distribution of *cphA*-related genes 93–95 % identical to *A. hydrophila cphA* genes (GenBank accession no. X57102) varied among *Aeromonas* species in the present study. *cphA*-related genes were noted in *A. veronii* and *A. hydrophila*, but not in *A. caviae*. Such results were concordant

Table 2. Presence of *cphA*-related genes (*cphA*), *in vitro* imipenem susceptibility studied with standard and high inocula, and the MHT in 51 blood isolates of different *Aeromonas* species

Blood isolates [<i>n</i> (% of 51 isolates)]	No. of isolates with <i>cphA</i> * (% of each species)	Imipenem-non-susceptible isolates† [<i>n</i> (% of each species)]				Positive MHT	
		Disc diffusion 0.5 McFarland		Etest 0.5 McFarland		Agar dilution	
		10 ⁴ c.f.u.		10 ⁷ c.f.u.		10 ⁷ c.f.u.	
<i>A. aquariorum</i> , 16 (31)	16 (100)	1 (6.3)	1 (6.3)	1 (6.3)	1 (6.3)	15 (94)	15 (94)‡
<i>A. veronii</i> , 12 (24)	12 (100)	0 (0)	0 (0)	0 (0)	0 (0)	12 (100)	12 (100)
<i>A. hydrophila</i> , 5 (10)	4 (80)	0 (0)	0 (0)	0 (0)	0 (0)	4 (80)	4 (80)
<i>A. caviae</i> , 15 (29)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)§
<i>Aeromonas</i> spp., 3 (6)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	3 (100)
Total, 51 (100)	35 (69)	1 (2)	1 (2)	1 (2)	1 (2)	34 (67)	34 (67)

*Detected by PCR.

†Imipenem-non-susceptible: an inhibition zone diameter <16 mm by disc diffusion test or an MIC >4 µg ml⁻¹ by Etest or agar dilution method.

‡The MHT result of an imipenem-susceptible isolate was indeterminate.

§The MHT results of two isolates were indeterminate.

with those in previous studies (Balsalobre *et al.*, 2009; Rossolini *et al.*, 1995). However, it is a novel finding that the *cphA*-related genes were present in all *A. aquariorum* isolates.

Determination of *in vitro* susceptibility to carbapenems by either the disc diffusion or dilution techniques among carbapenemase-producing *Aeromonas* isolates has often failed to yield a carbapenem-resistant phenotype (Massidda *et al.*, 1991; Rossolini *et al.*, 1996). Likewise, our *cphA*⁺ aeromonads rarely exhibited the imipenem-non-susceptible phenotype by the disc diffusion, Etest or agar dilution techniques with the recommended inoculum. However, with a higher inoculum, imipenem non-susceptibility was increasingly demonstrated among *cphA*⁺ isolates, and absent among *cphA*⁻ isolates. Among the detection methods for carbapenem resistance, the agar dilution test with large inocula was the most sensitive (97%). The only exception, *A. aquariorum* A2-155, was susceptible to imipenem even when tested with a large inoculum. Rossolini *et al.* (1995) found that 20% of *cphA* hybridized-positive *A. hydrophila* and *A. veronii* bv. *sobria* isolates were unable to express MBL activity, and they proposed that genetic modification had led to silencing of *cphA* in these species. Such a genetic modification was noted in A2-155, in which an extra inserted sequence in *cphA* might have altered the expression of CphA MBL. Owing to the failure of conventional *in vitro* susceptibility tests to detect carbapenemase production in aeromonads, we echo the advice of Rossolini *et al.* (1996) that it is prudent to consider *Aeromonas* species with carbapenemase-encoding genes as being resistant to carbapenems, unless they are verified to be unable to produce carbapenemase or to be carbapenem-susceptible by susceptibility testing using a large inoculum.

Similarly, the detection of *cphA* MBL activity, which could be inhibited by ion chelators, among aeromonads is inoculum-dependent. With the disc diffusion method and Etest, the zone diameters of inhibition increased and the MIC values decreased in the presence of EDTA among most *cphA*⁺ isolates when tested with large inocula. Other phenotypic methods to detect MBL are the MHT (Lee *et al.*, 2001) and synergy tests based on the ability of metal chelators (Arakawa *et al.*, 2000). However, even with large inocula, EDTA synergy disc tests and Etest detected MBL among only one-third and one-half, respectively, of *cphA*⁺ aeromonads in our study. The MHT is recommended for the detection of carbapenemases in members of the *Enterobacteriaceae* by the CLSI (CLSI, 2009). However, the sensitivity and specificity of the test for detecting isolates with low-level carbapenemase production, such as aeromonads, are not known (CLSI, 2009). In our observation, the MHT performed well at detecting carbapenemase activity in our *Aeromonas* isolates.

The clinical relevance of CphA MBL in *Aeromonas* species remains obscure. In the literature, the clinical outcome in patients receiving carbapenems for infections caused by

Table 3. Imipenem susceptibility, expression of MBL and the MHT in 51 *Aeromonas* blood isolates with or without *cphA*-related genes

Method (inoculum)	No. of isolates (%)	
	<i>cphA</i> ⁺ (n=35)	<i>cphA</i> ⁻ (n=16)
Imipenem non-susceptibility*		
Standard inocula		
Disc diffusion (0.5 McFarland)	1 (3)	0 (0)
Etest (0.5 McFarland)	1 (3)	0 (0)
Agar dilution (10 ⁴ c.f.u.)	1 (3)	0 (0)
High inocula		
Disc diffusion (1.5 McFarland)	12 (34)	0 (0)
Etest (1.5 McFarland)	17 (49)	0 (0)
Agar dilution (10 ⁷ c.f.u.)	34 (97)	0 (0)
Phenotypic expression of MBL		
Standard inocula (0.5 McFarland standard)		
Imipenem-EDTA combined disc	1 (3)	0 (0)
Imipenem-EDTA MBL Etest	1 (3)	0 (0)
High inocula (1.5 McFarland standard)		
Imipenem-EDTA combined disc	10 (29)	0 (0)
Imipenem-EDTA MBL Etest	17 (49)	0 (0)
Positive MHT with ertapenem disc	34 (97)	0 (0)

*Imipenem non-susceptibility: an inhibition zone diameter <16 mm by disc diffusion test or an MIC >4 µg ml⁻¹ by Etest or agar dilution method.

Fig. 1. Results of the MHT. Positive results for (a) *A. aquariorum* MDC47^T, (b) *A. hydrophila* ATCC 7966^T, (c) *A. aquariorum* A2-1000804 and (d) *A. aquariorum* A2-70; (e) a negative result for *A. caviae* A2-27; (f) an indeterminate result for *A. aquariorum* A2-155, in which the indentation (>) between the aeromonad streak and the margin of ertapenem inhibition zone was not obvious. →, Tested *Aeromonas* isolate, ♦, *Klebsiella pneumoniae* ATCC BAA-1705 as the positive control; ▼, *K. pneumoniae* ATCC BAA-1706 as the negative control; *Escherichia coli* ATCC 25922 was the indicator organism.

Aeromonas species with *cphA* has been rarely described, and both successful (Mukhopadhyay *et al.*, 2003) and fatal (Itoh *et al.*, 1999) outcomes have been reported. Theoretically, carbapenem monotherapy would fail to inhibit the growth of MBL-producing aeromonads in infectious diseases with high tissue bacterial burdens, such as peritonitis/abdominal sepsis or soft tissue infections. The bacterial loads of these infected sites might reach more than 10^7 c.f.u. ml⁻¹ (Brian & Julian, 2011; Mouës *et al.*, 2004), much higher than the inoculum of 10^4 c.f.u. ml⁻¹ for the standard agar dilution test. Moreover, the production of CphA was significantly increased in the presence of a suitable β -lactamase inducer, such as benzylpenicillin or imipenem (Segatore *et al.*, 1993). The emergence of an imipenem-resistant *Aeromonas* isolate during carbapenem treatment was noted in one of our patients and in a cirrhotic patient with *A. hydrophila* osteomyelitis (Lee *et al.*, 2003). The development of imipenem resistance in an *A. veronii* isolate with ImiS (98% identical to CphA) recovered from a patient with antecedent amoxicillin-clavulanate treatment has also been reported (Sánchez-Céspedes *et al.*, 2009). These observations highlight the controversy of carbapenem therapy for infectious diseases caused by *cphA*-carrying *Aeromonas* isolates. Therefore, we advise performing the susceptibility tests with a large inoculum or the MHT before considering a carbapenem-based chemotherapy for infections due to *Aeromonas* species.

In conclusion, the distribution of *cphA*-related genes among aeromonads is species-specific, found in *A. aquariorum*, *A. veronii* and *A. hydrophila*. However, *cphA*⁺ *Aeromonas* species rarely exhibited the imipenem-non-susceptible phenotype using *in vitro* susceptibility tests unless they were tested with a large inoculum. The MHT could be a screening test for CphA production in aeromonads before a carbapenem-based chemotherapy.

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