Distribution and phenotypic and genotypic detection of a metallo-\(\beta\)-lactamase, CphA, among bacteraemic Aeromonas isolates

Chi-Jung Wu,1,2,3 Po-Lin Chen,1,3 Jiunn-Jong Wu,4 Jing-Jou Yan,5 Chin-Chi Lee,3,6 Hsin-Chun Lee,3,6 Nan-Yao Lee,3,6 Chia-Ming Chang,3,6 Yu-Tzu Lin,2 Yen-Cheng Chiu3 and Wen-Chien Ko3,6

1Department of Graduate Institute of Clinical Medicine, National Cheng Kung University Medical College, Tainan, Taiwan, ROC
2National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Tainan, Taiwan, ROC
3Department of Internal Medicine, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan, ROC
4Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan, Taiwan, ROC
5Department of Pathology, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan, ROC
6Center for Infection Control, National Cheng Kung University Hospital, Tainan, Taiwan, ROC

The objectives of the study were to investigate the distribution of cphA-related genes (cphA) encoding a CphA metallo-\(\beta\)-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 (104 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 (107 c.f.u.), 34 (97 %) of 35 cphA+ isolates had imipenem MICs of \(\geq 16\) mg l\(^{-1}\), higher than the susceptible breakpoint (4 mg l\(^{-1}\)), and demonstrated positive results for the MHT, while one cphA+ and all 17 cphA- isolates had imipenem MICs of \(\leq 4\) mg l\(^{-1}\). 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.

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 \(\beta\)-lactamases have been recognized in Aeromonas species, i.e. Ambler class C cephalosporinases, class D penicillinas and class B...
CphA in bacteraemic Aeromonas isolates

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 (10⁶ 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 (Figuera et al., 2009), a recently described species found in aquarium water and ornamental fish (Martinez-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 (Figuera 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 etanopretin 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 β-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'-GACGAGAAGARTTCGTTGTTCC-3') and Rpsb-R (5'-GTGTGC-ATGTNGNACCAT-3') (Kupper 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 ≤ 0.9% for a given type strain were identified to that species (Lamy et al., 2010).

A. aquariorum MDC47T (=BCRC 17946T), A. veronii bv. sobria ATCC 9071T, A. hydrophila ATCC 7966T and A. caviae ATCC 13136T 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 (5'-GCTTAGACCTCCTGAAGGGAAGATGAAAATTG-3'); 5'-GCATAGTACCTTATGACTGGGGTGCGGCCTTG-3' (Lamy et al., 1993). Compared with other class B enzymes, CphA is inhibited by metal chelators, such as EDTA (Massidda et al., 1991; 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 (10⁶ 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 (Figuera et al., 2009), a recently described species found in aquarium water and ornamental fish (Martinez-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 (Figuera 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.
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 27853T, 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 ≤0.9% for the rpoB genes of A. aquariorum DSM 18362T (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 MDC47T 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 18362T was determined as A. aquariorum.

By PCR amplification and sequence analysis, A. aquariorum MDC47T, A. aquariorum A2-1000804, A. veronii bv. sobria ATCC 9071T, A. hydrophila ATCC 7966T 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 MDC47T and A2-1000804, A. veronii bv. sobria ATCC 9071T, A. hydrophila ATCC 7966T 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 MDC47T 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 blaIMP and blaVIM 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.
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 (Küpper 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 with the observation that *A. aquariorum* was the predominant species causing bacteraemia.
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 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

<table>
<thead>
<tr>
<th>Method (inoculum)</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>cphA</em>+ (<em>n</em>=35)</td>
</tr>
<tr>
<td><strong>Imipenem non-susceptibility</strong>&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Standard inocula</td>
<td></td>
</tr>
<tr>
<td>Disc diffusion (0.5 McFarland)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Etest (0.5 McFarland)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Agar dilution (10&lt;sup&gt;8&lt;/sup&gt; c.f.u.)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>High inocula</td>
<td></td>
</tr>
<tr>
<td>Disc diffusion (1.5 McFarland)</td>
<td>12 (34)</td>
</tr>
<tr>
<td>Etest (1.5 McFarland)</td>
<td>17 (49)</td>
</tr>
<tr>
<td>Agar dilution (10&lt;sup&gt;7&lt;/sup&gt; c.f.u.)</td>
<td>34 (97)</td>
</tr>
<tr>
<td><strong>Phenotypic expression of MBL</strong></td>
<td></td>
</tr>
<tr>
<td>Standard inocula (0.5 McFarland standard)</td>
<td></td>
</tr>
<tr>
<td>Imipenem-EDTA combined disc</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Imipenem-EDTA MBL Etest</td>
<td>1 (3)</td>
</tr>
<tr>
<td>High inocula (1.5 McFarland standard)</td>
<td></td>
</tr>
<tr>
<td>Imipenem-EDTA combined disc</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Imipenem-EDTA MBL Etest</td>
<td>17 (49)</td>
</tr>
<tr>
<td><strong>Positive MHT with ertapenem disc</strong></td>
<td></td>
</tr>
</tbody>
</table>

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

**Fig. 1.** Results of the MHT. Positive results for (a) *A. aquariorum* MDC47<sup>T</sup>, (b) *A. hydrophila* ATCC 7966<sup>T</sup>, (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; Moués 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.

ACKNOWLEDGEMENTS

This study was supported by the National Science Council, Taiwan (grants NSC 99-2628-B-006-014-MY3 and NSC 98-2320-B-006-029); Department of Health, Executive Yuan (DOH100-TD-B-111-002); and the National Health Research Institutes (id-100-pp-17). We thank Miss Hui-Ju Hung and Pei-Chen Wu for the laboratory work.

REFERENCES


