BMC
Infectious Diseases

RESEARCH ARTICLE　　　　　　　　　　　　　　　　　　　**Open Access**

Dissemination of imipenem-resistant Acinetobacter baumannii with new plasmid-borne bla$_{OXA-72}$ in Taiwan

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Abstract

Background: The systemic surveillance of imipenem-resistant *Acinetobacter baumannii* (IRAB) from multicenters in Taiwan revealed the emergence of isolates with bla$_{OXA-72}$. This study described their genetic makeup, mechanism of spread, and contribution to carbapenem resistance.

Methods: Two hundred and ninety-one non-repetitive isolates of *A. baumannii* were collected from 10 teaching hospitals from different geographical regions in Taiwan from June 2007 to September 2007. Minimal inhibitory concentrations (MICs) were determined by agar dilution. Clonality was determined by pulsed-field gel electrophoresis. Plasmid was extracted and digested by restriction enzymes, and subsequently analyzed by electrophoresis and Southern blot for bla$_{OXA-72}$. The flanking regions of bla$_{OXA-72}$ were determined by inverse PCR. The contribution of bla$_{OXA-72}$ to imipenem MIC was determined by transforming plasmids carrying bla$_{OXA-72}$ into imipenem-susceptible *A. baumannii*.

Results: Among 142 IRAB in Taiwan, 27 harbored bla$_{OXA-72}$; 22 originated from Southern Taiwan, 5 from Central Taiwan, and none from Northern Taiwan. There were two major clones. The bla$_{OXA-72}$ was identified in the plasmids of all isolates. Two genetic structures flanking plasmid-borne bla$_{OXA-72}$ were identified and shared identical sequences in certain regions; the one described in previous literature was present in only one isolate, and the new one was present in the remaining isolates. Introduction of bla$_{OXA-72}$ resulted in an increase of imipenem MIC in the transformants. The overexpression of bla$_{OXA-72}$ mRNA in response to imipenem further supported the contribution of bla$_{OXA-72}$.

Conclusions: In conclusion, isolates with new plasmid-borne bla$_{OXA-72}$ were found to be disseminated successfully in Southern Taiwan. The spread of the resistance gene depended on clonal spread and dissemination of a new plasmid. Bla$_{OXA-72}$ in these isolates directly led to their imipenem-resistance.

Keywords: Imipenem-resistant, *Acinetobacter baumannii*, Carbapenemase, Bla$_{OXA-72}$

Background

Acinetobacter baumannii has become an important nosocomial pathogen throughout the world because of its drug resistant phenotype [1]. The resistance to carbapenems is the most troublesome due to a limited number of efficacious drugs and the association with poor prognosis [2]. The mortality associated with imipenem-resistant *A. baumannii* (IRAB) is higher than that caused by susceptible strains, which is attributable to the higher rate of inappropriate antimicrobial therapy [3,4]. Although modification of antibiotic targets, overexpression of efflux pumps, and loss of porins leads to carbapenem resistance, the main mechanism in the *Acinetobacter* species is production of carbapenemases, including Ambler class B metallo-β-lactamases and class D β-lactamases [5]. Compared with other *Acinetobacter* species, class D β-lactamases in *A. baumannii*, including the intrinsic bla$_{OXA-51-like}$ as well as acquired bla$_{OXA-23-like}$, bla$_{OXA-24-like}$, bla$_{OXA-58-like}$, and bla$_{OXA-143}$ genes are more commonly identified as the cause of resistance [5,6]. The

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distribution of class D β-lactamases varies among countries, even among regions in the same country [7]. Isolates with bla_{OXA-23} spread over a wide geographical range [8], whereas those carrying bla_{OXA-24}, though relatively less common, have emerged in France, Italy, South Korea, and Brazil [9-12]. In Taiwan, one pilot study described the spread of isolates with bla_{OXA-72}, a member of the bla_{OXA-24} family, within a single medical center [13]. Thus, in this study, we collected clinical isolates of *A. baumannii* from 10 medical centers in different areas in Taiwan during 2007 and found the different geographic distribution of isolates carrying $bla_{OXA-24/72}$. Therefore, this multicenter study aimed to describe their genetic makeup, mechanism of spread, and contribution to carbapenem resistance in *A. baumannii* in Taiwan.

Methods

Bacterial identification and antimicrobial susceptibility testing

Two hundred and ninety-one non-repetitive isolates of *A. baumannii* were retrospectively collected from 10 teaching hospitals from different geographical regions in Taiwan from June 2007 to September 2007. Four hospitals are located in Northern (N1-N4) Taiwan, 3 are located in Central (C1-C3) Taiwan, and 3 are located in Southern (S1-S3) Taiwan (Additional File 1). All isolates were taken as part of standard patient care and investigators retrospectively collected these isolates. This microbiological study without patient data was exempt from full review by our institutional review boards.

All isolates were identified to the species level using a multiplex PCR method to confirm the specific intergenic spacer region in *A. baumannii* [14]. The minimal inhibitory concentration (MIC) of imipenem, meropenem, ticarcillin, piperacillin, ceftazidime, cefepime, sulbactam, ciprofloxacin, and colistin was determined using the agar dilution method according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [15]. The testing and interpretation were in accordance with the guidelines of CLSI breakpoints or manufacturer's instructions. *A. baumannii* isolates that were resistant to imipenem were subjected to the experiments described below.

Identifying Ambler class B metallo-β-lactamases and class D β-lactamases

PCR methods were used to detect $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-58-like}$, $bla_{ISAba1-OXA51-like}$, $bla_{OXA-143}$, $bla_{IMP-like}$, $bla_{VIM-like}$, bla_{GIM-1}, bla_{SPM-1} and bla_{SIM-1} as previously described [6,16,17]. The PCR products were then sequenced at Mission Biotech, Taipei, Taiwan. Primers of ISAba1F and ISAba1R were used to detect the presence of ISAba1, whereas the ISAba1F and OXA-likeR primers against the different genes described above were used to confirm the presence of ISAba1 upstream of the carbapenemase gene [16].

Determination of clonality by pulsed-field gel electrophoresis of isolates with bla_{OXA-72}

The clonality of strains with bla_{OXA-72} was determined by pulsed-field gel electrophoresis after digestion with *Apa*I as previously described [18]. The stained gel was photographed and analyzed by BioNumerics software (Applied Maths) to generate a dendrogram of relatedness among these isolates. Isolates with similarity > 85% was designated as one clone.

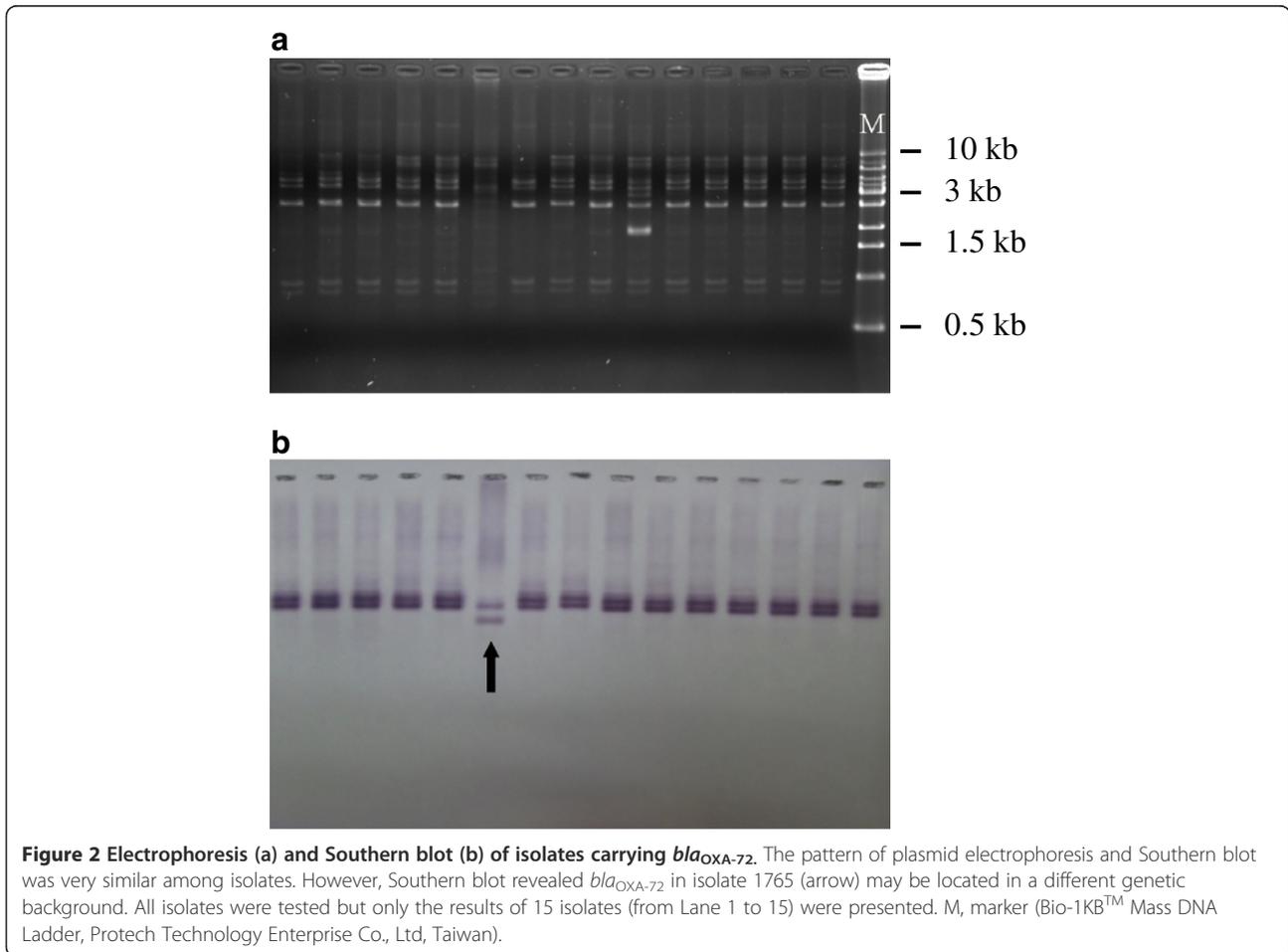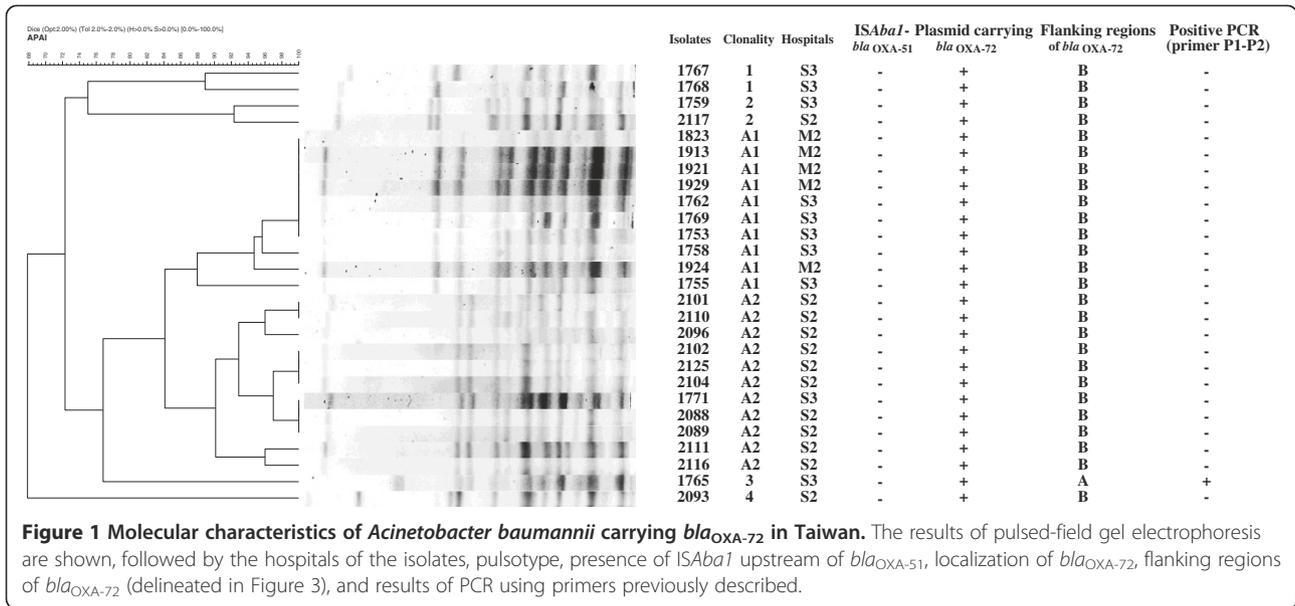### Determination of the plasmid localization of bla_{OXA-72} by Southern blot

The plasmid localization of bla_{OXA-72} was visualized as previous reported [19]. The plasmid was extracted with a plasmid DNA Miniprep Kit (Bioman, Taipei, Taiwan) or a plasmid Maxiprep Kit (Qiagen, Valencia, CA). The extracts were treated with *Eco*RI and subjected to electrophoresis. After transfer to the hybridization transfer membrane (PerkinElmer, Boston, MA) and hybridization with a PCR-generated probe derived from primers targeting bla_{OXA-72} (Additional File 2), the band was visualized with a digoxigenin (DIG) DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Sequencing of flanking region of plasmid-borne bla_{OXA-72} by inverse PCR

Inverse PCR was performed as previously reported [20]. Briefly, the plasmid extracted from isolates carrying bla_{OXA-72} was digested with *Eco*RI, *Hin*dIII, or *Bgl*II, respectively. After overnight incubation and subsequent purification, T4 DNA ligase (Promega Corp., Madison, USA) was added in each extract according to the manufacturer's instructions. Outward amplification with PCR primers (Additional File 2) covering portions of the bla_{OXA-72} region was carried out under standard con-

Table 1 Minimal inhibitory concentrations (MICs) of antimicrobial agents for *Acinetobacter baumannii* carrying bla_{OXA-72}

Antimicrobial agents	Isolates carrying bla_{OXA-72} ($n = 27$)		
	MIC50 (mg/L)	MIC90 (mg/L)	Resistance rate (%)
Imipenem	128	128	100.0%
Meropenem	>128	>128	100.0%
Ticarcillin	>128	>128	100.0%
Piperacillin	>128	>128	100.0%
Ceftazidime	>128	>128	100.0%
Cefepime	64	>128	96.3%
Sulbactam	32	32	63.0%
Ciprofloxacin	128	>128	100.0%
Colistin	1	1	0.0%

Isolates	Clonality	Hospitals	ISAba1- bla OXA-51	Plasmid carrying bla OXA-72	Flanking regions of bla OXA-72	Positive PCR (primer P1-P2)
1767	1	S3	-	+	B	-
1768	1	S3	-	+	B	-
1759	2	S3	-	+	B	-
2117	2	S2	-	+	B	-
1823	A1	M2	-	+	B	-
1913	A1	M2	-	+	B	-
1921	A1	M2	-	+	B	-
1929	A1	M2	-	+	B	-
1762	A1	S3	-	+	B	-
1769	A1	S3	-	+	B	-
1753	A1	S3	-	+	B	-
1758	A1	S3	-	+	B	-
1924	A1	M2	-	+	B	-
1755	A1	S3	-	+	B	-
2101	A2	S2	-	+	B	-
2110	A2	S2	-	+	B	-
2096	A2	S2	-	+	B	-
2102	A2	S2	-	+	B	-
2125	A2	S2	-	+	B	-
2104	A2	S2	-	+	B	-
1771	A2	S3	-	+	B	-
2088	A2	S2	-	+	B	-
2089	A2	S2	-	+	B	-
2111	A2	S2	-	+	B	-
2116	A2	S2	-	+	B	-
1765	3	S3	-	+	A	+
2093	4	S2	-	+	B	-

Figure 1 Molecular characteristics of *Acinetobacter baumannii* carrying *bla*_{OXA-72} in Taiwan. The results of pulsed-field gel electrophoresis are shown, followed by the hospitals of the isolates, pulsotype, presence of IS*Aba1* upstream of *bla*$_{OXA-51}$, localization of *bla*$_{OXA-72}$, flanking regions of *bla*$_{OXA-72}$ (delineated in Figure 3), and results of PCR using primers previously described.

Figure 2 Electrophoresis (a) and Southern blot (b) of isolates carrying *bla*$_{OXA-72}$. The pattern of plasmid electrophoresis and Southern blot was very similar among isolates. However, Southern blot revealed *bla*$_{OXA-72}$ in isolate 1765 (arrow) may be located in a different genetic background. All isolates were tested but only the results of 15 isolates (from Lane 1 to 15) were presented. M, marker (Bio-1KB^{TM} Mass DNA Ladder, Protech Technology Enterprise Co., Ltd, Taiwan).

ditions. The product was then sent for sequencing at Mission Biotech, Taipei, Taiwan.

Determination of the contribution of bla_{OXA-72} to carbapenem MIC

The bla_{OXA-72} was cloned into the shuttle vector pYMAb2 [21], which carries a kanamycin resistant determinant. The recombinant plasmid (pYMAb2:: bla_{OXA-72}) and control plasmid (pYMAb2) were then transformed into the kanamycin-susceptible Ab290 strain by electroporation using an gene pulser electroporator (Bio-Rad, Hercules, CA) and 2 mm electrode gap cuvettes. The antimicrobial MICs of Ab290 (pYMAb2:: bla_{OXA-72}) and Ab290 (pYMAb2) were determined as previously described [15]. Ab290 was an *A. baumannii* clinical isolate that was susceptible to multiple antimicrobial agents from Taipei Veterans General Hospital and has been used for transformation in the previous study [19]. After incubation without or with antimicrobial agents, the OXA-72 mRNAs in Ab290 (pYMAb2:: bla_{OXA-72}) were compared using quantitative PCR [21]. Briefly, bacterial RNA was extracted using an RNAprotect Bacteria Reagent and RNeasy mini-kit (Qiagen, Valencia, CA). After elimination of genomic DNA by RNase-free DNase (Qiagen) treatment, reverse transcription was performed using random hexamers (Qiagen). Real-time PCR for bla_{OXA-72} was carried out with *recA* as an internal control on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Inc.) These experiments were performed in at least triplicate.
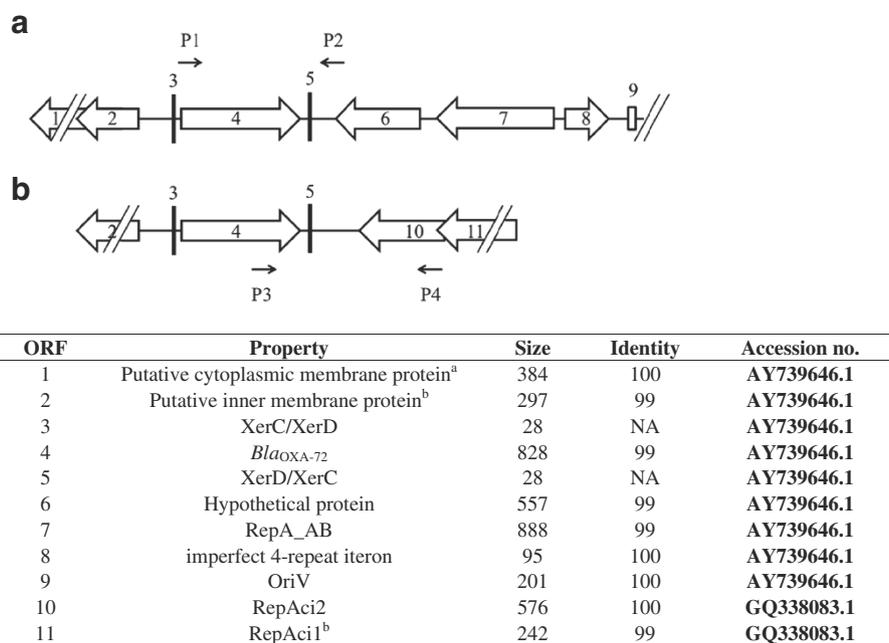
Results

Bacterial identification and antimicrobial susceptibility testing

One-hundred and forty-two *A. baumannii* strains were resistant to imipenem. Among them, 27 (19.0%) carried bla_{OXA-72}, including 22 of 38 isolates from Southern Taiwan (57.9%) and 5 of 62 isolates from Central Taiwan (8.1%). The number of isolates with bla_{OXA-72} was significantly higher in those obtained from the southern area than those from other areas (57.9% vs. 4.8%, respectively; $P < 0.001$ by χ^2 test). All of the *A. baumannii* isolates carrying bla_{OXA-72} were resistant to carbapenems (Table 1). In addition, they were all multidrug resistant [22], but were susceptible to colistin.

Identifying Ambler class B metallo-β-lactamases and class D β-lactamases

All isolates with bla_{OXA-72} had the $bla_{OXA-51-like}$. The $bla_{OXA-23-like}$, $bla_{OXA-58-like}$, $bla_{OXA-143}$, $bla_{IMP-like}$, $bla_{VIM-like}$, bla_{GIM-1}, bla_{SPM-1} and bla_{SIM-1} genes were not detected by PCR methods. In addition, ISAba1 was not detected in any of the strains.

ORF	Property	Size	Identity	Accession no.
1	Putative cytoplasmic membrane protein[a]	384	100	**AY739646.1**
2	Putative inner membrane protein[b]	297	99	**AY739646.1**
3	XerC/XerD	28	NA	**AY739646.1**
4	Bla_{OXA-72}	828	99	**AY739646.1**
5	XerD/XerC	28	NA	**AY739646.1**
6	Hypothetical protein	557	99	**AY739646.1**
7	RepA_AB	888	99	**AY739646.1**
8	imperfect 4-repeat iteron	95	100	**AY739646.1**
9	OriV	201	100	**AY739646.1**
10	RepAci2	576	100	**GQ338083.1**
11	RepAci1[b]	242	99	**GQ338083.1**

[a] Partial sequence for plasmid A; [b] Partial sequence for plasmid B

Figure 3 Schematic map of the partial flanking region of bla_{OXA-72} in two types of plasmids (plasmid A and B, Figures 3a and 3b, respectively) in Taiwan. The arrows indicate the predicted open reading frames (ORFs) and their transcriptional directions. The names of various features are indicated below the map. The sequenced fragment in plasmid A shared 99% similarity to the previously prevalent plasmid (accession no. **AY739646.1**), whereas the region downstream of XerD/XerC in plasmid B was identical to another plasmid (accession no. **GQ338083.1**). P1 and P2 primers are specific for plasmid A and P3 and P4 primers are specific for plasmid B.

Table 2 Minimal inhibitory concentrations (mg/L) of antimicrobial agents for *Acinetobacter baumannii* with or without plasmids carrying *bla*$_{OXA-72}$

Isolates	Imipenem	Meropenem	Ticarcillin	Ampicillin	Sulbactam	Cefepime	Ceftazidime	Ciprofloxacin
Ab290	0.25	0.5	8	128	2	2	2	0.5
Ab290 (pAbYM2)	0.25	0.5	8	128	4	2	4	0.25
Ab290 (pAbYM2::*bla*$_{OXA-72}$)	32	32	>1024	>1024	16	4	4	0.5

Determination of clonality by pulsed-field gel electrophoresis

To determine whether emergence of these isolates with *bla*$_{OXA-72}$ was due to clonal spread, pulsed-field gel electrophoresis was performed (Figure 1). Twenty-one isolates (77.8% belonged to 2 major clones (A1 and A2) (Figure 1). The other six isolates carrying *bla*$_{OXA-72}$ (including isolate 1765) did not belong to the two major clones and had only 65-75% homology related to the major clones. The A1 clone comprised all (5/5, 100%) of the isolates from Central Taiwan and 5 isolates (5/10, 50.0%) from S3 hospital, whereas the A2 clone comprised 10 isolates (10/12, 83.3%) from S2 and 1 isolate (1/10, 10%) from S3 hospital.

Determination of the plasmid localization of *bla*$_{OXA-72}$ by Southern blot

Electrophoresis showed minor differences in plasmid patterns among isolates (Figure 2). Southern blot using a *bla*$_{OXA-72}$ probe further revealed the presence of *bla*$_{OXA-72}$ in the plasmids obtained from 27 isolates. However, Southern blot also detected two types of segments flanking *bla*$_{OXA-72}$ after *Eco*RI digestion. One was present in isolate 1765 and the other was present in the remaining 26 isolates.

Sequencing of the flanking region of plasmid-borne *bla*$_{OXA-72}$ by inverse PCR

Based on the Southern blot results, we anticipated that there were at least two genetic structures flanking the *bla*$_{OXA-72}$ gene. Inverse PCR of isolate 1765 and randomly selected isolates revealed two types of genetic structures flanking the gene (Figures 3a and 3b, fragment A of 3596 bp and B of 2325 bp). An identical 967 bp region containing *bla*$_{OXA-72}$ in both fragment A and B was flanked by XerC/XerD and XerD/XerC. The 3596 bp fragment A had 99% similarity to pAB02 (GenBank accession no. **AY228470.1**) and contained a region with 100% similarity to the plasmids reported from the same teaching hospital (828 bp, accession no. **AY739646.1**) [13]. In fragment B, the 1195 bp region upstream of XerD/XerC had 99% similarity to that in fragment A, but the region downstream of XerD/XerC was similar to other plasmids without *bla*$_{OXA-72}$ (accession no. **GQ338083.1**), indicating possible recombination between the plasmids. Using a specific primer pair (P3 and P4, Additional File 2) for fragment B (Figure 3), the PCR result was positive in 26 isolates, except isolate 1765. The specific primers used to
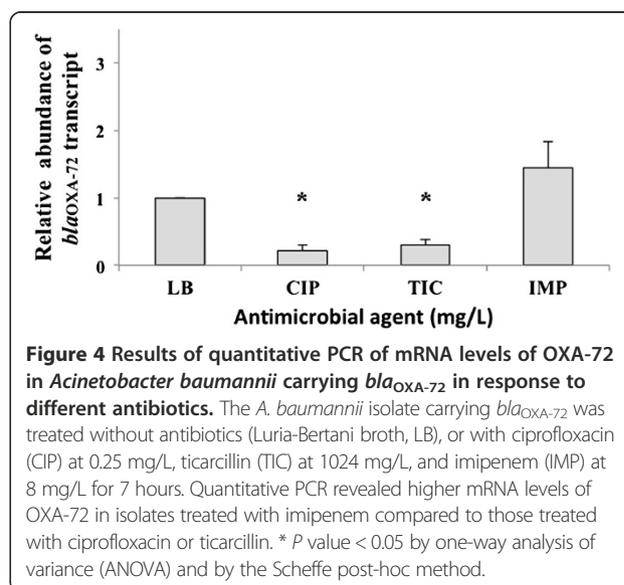
confirm the presence of fragment A were similar to those used in a previous study (primers P1 and P2, respectively; Figure 3) [13]. Only one isolate (isolate 1765) was positive for fragment A. The mutual exclusion of fragment A and B indicated that they were located on different plasmids, designated as plasmid A and B, respectively.

Determination of the contribution of *bla*$_{OXA-72}$ to carbapenem MIC

To determine the contribution of *bla*$_{OXA-72}$, *A. baumannii* strain Ab290 was transformed with pYMAb2 and pYMAb2::*bla*$_{OXA-72}$, respectively. Compared to Ab290 (pYMAb2), transformation with *bla*$_{OXA-72}$ resulted in an increase in the MICs of imipenem, meropenem, ticarcillin, sulbactam, and ampicillin (Table 2). We were interested in determining if the addition of carbapenems would increase OXA-72 expression. Thus, strain Ab290 (pYMAb2:: *bla*$_{OXA-72}$) was treated with ciprofloxacin, imipenem, and ticarcillin. Quantitative PCR showed that the RNA expression of the gene increased by 8-fold after addition of imipenem compared to addition of ciprofloxacin or ticarcillin (Figure 4).

Discussion

The resistance rate to carbapenems in *A. baumannii* has been increasing worldwide. Ambler class D β-lactamases

Figure 4 Results of quantitative PCR of mRNA levels of OXA-72 in *Acinetobacter baumannii* carrying *bla*$_{OXA-72}$ in response to different antibiotics. The *A. baumannii* isolate carrying *bla*$_{OXA-72}$ was treated without antibiotics (Luria-Bertani broth, LB), or with ciprofloxacin (CIP) at 0.25 mg/L, ticarcillin (TIC) at 1024 mg/L, and imipenem (IMP) at 8 mg/L for 7 hours. Quantitative PCR revealed higher mRNA levels of OXA-72 in isolates treated with imipenem compared to those treated with ciprofloxacin or ticarcillin. * *P* value < 0.05 by one-way analysis of variance (ANOVA) and by the Scheffe post-hoc method.

are responsible for the main mechanism of resistance in *A. baumannii*. Bla$_{OXA-72}$, which was first discovered in Thailand, has since spread rapidly to other areas of Asia and Europe [9-13]. In this study we collected 142 IRAB from different geographical regions of Taiwan. Among them, 27 IRAB carrying bla_{OXA-72} gene had successfully spread throughout Southern Taiwan and extended into Central Taiwan. One new type of plasmid, which harbored a different genetic structure compared to that previously reported, not only existed in the two major clones, but also disseminated to other clones. We also found evidence that bla_{OXA-72} contributed to carbapenem resistance. The mRNA levels of OXA-72 increased in response to the addition of imipenem.

Many studies have demonstrated an association of carbapenem resistance and the presence of bla_{OXA-72}. One study [23] revealed that the plasmid carrying bla_{OXA-72} modestly increased the MICs of imipenem and meropenem in *Escherichia coli* (0.125 to 0.25 and 0.016 to 0.032, respectively). Another study [9] showed that transformation of susceptible strains with the entire plasmid carrying bla_{OXA-72} extracted from clinical isolates resulted in resistance to carbapenems, but not cephalosporins. However, the increase of carbapenem MIC might be attributed to other genetic determinants also present in the transformed plasmid in that study. In our study, we only cloned the bla_{OXA-72} gene into the plasmid without other resistance mechanisms against carbapenems. The transformation greatly increased the MICs of carbapenems (64-128–fold), which was further supported by the elevated mRNA levels of OXA-72 in response to the addition of imipenem. Moreover, our study has revealed for the first time that the transformation also increased the MICs of ticarcillin, ampicillin, and sulbactam.

The molecular epidemiology of Ambler class D β-lactamases differs among geographical regions [7]. Our previous study showed prevalence of bla_{OXA-23} in Central Taiwan [24], whereas the current study showed the bla_{OXA-72} was mainly disseminated in Southern Taiwan. Our study revealed that the clonal spread and plasmid dissemination were important for bla_{OXA-72}, similar to previous studies [13,23,25]. The mechanisms of spread of different carbapenemase genes seemed to be different, as our previous study showed that a transposon may be the main reason of spread of bla_{OXA-23} in Central Taiwan [24].

Lu *et al.* found that the plasmid carrying bla_{OXA-72} was positive for PCR in half of the isolates (49.2%, 29/59) in one teaching hospital in Southern Taiwan using P1 and P2 primers [13]. Our study recovering isolates from multiple centers only identified one isolate from the same hospital assessed in a previous study [13] that may harbor the same plasmid (plasmid A). This suggests that plasmid A may have been replaced by plasmid B during this period. Plasmid B shared part of the genetic structure with plasmid A.

The shared regions included not only regions between XerC/XerD binding sites, which were presumed to be responsible for mobilization of bla_{OXA-72} [23,25,26], but also regions upstream from it. This may suggest that these plasmids have undergone a recombination event.

Conclusions
In conclusion, our study has revealed that the dissemination of IRAB isolates carrying the bla_{OXA-72} gene may be due to clonal spread and dissemination of a new plasmid. The bla_{OXA-72} gene contributed to carbapenem resistance.

Additional files

Additional file 1: Distribution of *Acinetobacter baumannii* carrying blaOXA-72 among 291 *A. baumannii* isolates collected from 10 teaching hospitals in Taiwan (N, northern; C, central; S southern).

Additional file 2: Primers used in this study.

Abbreviations
CLSI: Clinical and laboratory standards institute; MIC: Minimal inhibitory concentration; IRAB: Imipenem-resistant *Acinetobacter baumannii*.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SCK and SPY conceived of the study, and participated in its design and coordination and drafted the manuscript. YTL and HCC participated in the sequence alignment, and drafted the manuscript. CPC and CLC carried out the laboratory assays. PLL and PRH collected the clinical isolates. TLC and CPF designed and supervised the study, participated in data analysis and interpretation, and finalized the manuscript. All authors read and approved the final version of the manuscript.

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