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Full Length Research Paper

AdeABC efflux pump: Less important role in *Acinetobacter baumannii* against carbapenems

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AdeABC efflux pump in a total of 50 *Acinetobacter baumannii* strains were investigated and the role in contributing to hydrolysis carbapenems were further analyzed. All strains were divided into 3 groups according to their susceptibilities to Imipenem, Amikacin, Minocycline and Levofloxacin: Group A (22 imipenem-resistant strains), B (13 isolates which were imipenem-sensitive but resistant to at least one of the other three antibiotics) and C (15 isolates, sensitive to all the antimicrobials). Five gene types were observed according to REP-PCR and 39 isolates were included in the main one. Only one isolate (A2) was positive for efflux pump phenotype. All strains were positive for blaOXA51-like and AdeB gene and negative for blaOXA24, blaOXA58, VIM, IPM, and SIM-1. blaOXA23 were detectable merely in the 22 imipenem-resistant strains. 24 isolates from the same REP type representing for the 3 groups were selected for quantitative analysis of adeB expression. Compared with the mean level of Group B, only A2 expressed apparently higher (2.2 fold). The induction effects of imipenem and meropenem were analyzed as well. Compared with their freely grew isogenic counterparts, up regulated expression was observed only in 2 isolates under the pressure of imipenem and none in all under meropenem. These data indicate that AdeABC efflux pump play a less important role in *A. baumannii* against carbapenems.

Key words: *Acinetobacter baumannii*, Carbapenem resistance, AdeABC efflux pump, adeB gene.

INTRODUTION

Acinetobacter baumannii is increasingly an important opportunistic nosocomial pathogen, especially in the intensive care units (ICUs), and can cause various infections, including pneumonia, urinary tract infection and septicemia (Peleg et al., 2008; Wieczorek et al., 2008). Carbapenems were thought to be the last useful agents that could combat severe *A. baumannii* infections. But in the recent 20 years, carbapenem- resistant *Acinetobacter baumannii* isolates were reported with an ever faster frequency throughout the world (Peleg et al., 2008; Zhou et al., 2007; Coelho et al., 2006). OXA and MBL type carbapenemases were thought to be the main causes of the high resistance in *A. baumannii* (Walther-Rasmussen and Hoiby, 2006; Walsh et al., 2005). OXA-23-type carbapenemase, especially, is the most wide spread and powerful carbapenemase in *A. baumannii* against carbapenems (Peleg et al., 2008; Zhou et al., 2007; Walther-Rasmussen and Hoiby, 2006). It is thought that the AdeABC efflux pump play a significant role in *A. baumannii* against antimicrobial as well (Wieczorek et al., 2008; Magnet et al., 2001; Ruzin et al., 2007). AdeABC efflux pump, belonging to the resistance–nodulation-cell division family (RND) efflux pump, has a broad range of substrates, such as aminoglicosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluorquinolones, tigecycline and so on (Wieczorek et al., 2008; Ruzin et al., 2007). The pump has been divided into two types: inductive and constitutive, depending on whether inducible by the substrate or not (Wieczorek et al., 2008; Roberts et al., 1996). Although, meropenem resistance caused by the high expression of the efflux pump in *A. baumannii* has been reported by Huang and his colleagues (Huang et al., 2008), the role of AdeABC

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efflux pump in *A. baumannii* against carbapenems is still arguing (Bratu et al., 2008).

Thus, the appearance and expression of AdeABC efflux pump in a total of 50 *A. baumannii* strains, isolated from a tertiary medical center in China, were observed in this study. We describe here the role of AdeABC efflux pump in *A. baumannii* against carbapenems.

METHODS

Bacterial isolates

Fifty non-repetitive *A. baumannii* strains were isolated during March 2008 and March 2009 in a tertiary medical center in China. All isolates were assigned to Acinetobacter baumannii by Vitek GNI card (bioMe´rieux) and confirmed through the intrinsic bla$_{OXA-51-like}$ gene to the species (Turton et al., 2006).

Susceptibility testing

The susceptibility of Imipenem (Oxiod), Amikacin (Oxiod), Minocycline (Oxiod) and Levofloxacin (Oxiod) for all these isolates were determined by disk dilution method on M-H agar plates following CLSI guidelines. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Results were interpreted according to CLSI recommendations (CLSI, 2007).

REP-PCR

To characterize the 50 isolates genetically, Repetitive Extragenic Palindromic Sequence-Based PCR (REP-PCR) was performed with the primer pair REP1 5'-IIIGCGCCGICATCAGGC-3'and REP2 5'-ACGTCTTATCAGGCCTAC- 3', as described previously (Bou et al., 2000). Strains belonging to the same clones showing identical or highly similar profiles (up to two bands variations). Discrepancy in the intensity of bands was not taken into account.

Detection of efflux pump phenotype

The minimum inhibition concentrations (MICs) of Imipenem of the 22 carbapenems-resistant *A. baumannii* isolates were obtained through broth dilution method following CLSI guidelines (CLSI, 2007). Meanwhile, additional CCCP (sigma) with the final concentration of 5 μg /ml was added to the broth in another group. Isolates with two-four fold MIC reduction of Imipenem with the addition of CCCP were considered as positive for AdeABC efflux pump. The interference of CCCP was examined by the growth in antibiotic free broth.

Detection of carbapenemases gene and AdeB gene

Genomic DNAs for PCR were prepared by boiling method. Carbapenemases genes: IPM, VIM, SIM-1, bla$_{OXA-23}$, bla$_{OXA-24}$, bla$_{OXA-58}$, bla$_{OXA-51-like}$ and AdeB were screened in all isolates. The regulators of AdeABC efflux pump of A2, AdeRS two-component system, were detected as well. PCRs were performed in a pre-mix PCR system purchased from SBS Gentech with the primers and parameters described previously (Zhou et al., 2007; Turton et al., 2006; Jeon et al., 2005; Lee et al., 2005; Marchand et al., 2004). PCR products were analyzed by agarose gel electrophoresis. All amplicons from A2 were sequenced by invitrogen (Shanghai) at the same time. Blasts were performed in NCBI. The positive control was generously provided by Professor YunSong Yu, first affiliate Hospital of Zhejiang University.

Real-time reverse transcript PCR

24 isolates with the same REP type, which represent for the 3 groups, were selected for the quantitative analysis of adeB expression. Total RNA was extracted from the bacterial cells grown on M-H agar plate over night with RNA isolation kit (SBS Genetech, Shanghai) following the manual. For inductive purpose, imipenem (Oxiod) and meropenem (Oxiod) contained disc were added in a parallel experiment and total RNA was isolated with cells growing within 5 mm from the edge of the discs. RNA concentration and purity were assessed spectrophotometrically at wavelengths of 260 and 280 nm. The purity was further confirmed by amplifying AdeB gene with the total RNA as template through polymerase chain reaction. Real-time RT-PCR was carried out with reverse transcription kit (TaKaRa Biotech, Dalian) according to the manufacturer's instructions on ABI7500 Real-Time PCR system. Each reaction mixture (20 μL) contained approximate 10 ng total RNA. RT-PCR condition was programmed as the kit recommended. The primers and probes (Table 1) used in this study were synthesized by SBS Genetech, Shanghai. 16S rRNA of *A. baumannii* was conducted in parallel as housekeeping. The samples and standards were determined in triplicate.

RESULTS

Antibiotic susceptibility

Among the 50 *A. baumannii* isolates, 22 were imipenem-resistant (inhibition zone<13 mm). Resistance to amikacin, minocycline and levofloxacin were 35, 33, and 35 isolates, respectively. Susceptibility with an intermediate result was assigned as resistant. According to these results, all the strains were separated into three groups. Group A consisted of the 22 imipenem-resistant strains. Group B contained 13 isolates which were imipenem-sensitive but resistant to at least one of the other three antibiotics. The left 15 isolates, susceptible to all of the antimicrobial, were sent into Group C.

REP-PCR typing

Five types were observed among the 50 strains through agarose gel electrophoresis with the amplified fragments. The main group containing 39 isolates consisted of the 22 imipenem -resistant isolates, 9 of B and 8 of C. Isolates contained in the left 4 types were no more than 5 each. The typing profiles of all the 39 strains showed identical bands with the variation only in the intensity.

Efflux pump phenotype

All isolates grew well in imipenem-free 5 μg /ml CCCP broth. The addition of CCCP led to a fourfold reduction of

Table 1. Primers and Taqman probes used in quantitative real-time RT-PCR

Primer	5'-3' sequence	Size(bp)	Reference
16sRNA	F: GTAGCGGTGAAATGCGTAGA R: CTTTCGTACCTCAGCGTCAG Probe: CGAAGGCAGCCATCTGGCCT	85	16
adeB	F: CCAGAGGAAGATCAAGGTTGGT R: TTTTACATCGGGATTGTCTTTCAA Probe: CATGACTTCGTTCCAGCTACCTTCAGATGC	123	18

the imipenem MIC in only one isolate (A2). No change in MIC was observed in the other 21 imipenem-resistant isolates.

Carbapenemases of *A. baumannii*

$bla_{OXA-51-like}$ gene was positive in all the 50 strains with an expected size as well as adeB. bla_{OXA-23} gene was detectable only in the 22 carbapenems-resistant strains with the size of 501 bp. IPM, VIM, SIM-1, bla_{OXA-24}, bla_{OXA-58} were negative in all the 50 isolates. Sequencing studies carried on amplicons of $bla_{OXA-51-like}$, bla_{OXA-23}, adeB, adeR and adeS of A2 resulted in 100% identical with previously reported genes (GenBank NO.: AY750909, AJ132105, DQ294294, AF370885 and AF370885, respectively).

adeB expression

Relative adeB mRNA expression level to its own 16sRNA was 0.63 ± 0.015 (C.V = 2.38%) in Group B. Decreased expression was observed both in Group A and C compared with Group B, which were statistically significant ($P < 0.05$). Only one isolates (A2) showing increased expression which was 2.2 fold to the mean level of Group B. Increased expression of adeB were obtained in A1 and A3 under the pressure of imipenem, approximately 9.1 and 4.2 fold higher contrasted with their freely grew isogenic counterparts on M-H agar. No up-regulated expression was measured under the pressure of meropenem in all the 8 isolates. (Table 2)

DISCUSSION

Nosocomial outbreaks caused by carbapenems-resistant Acinetobacter baumannii have been reported worldwide. OXA-23, OXA-24, OXA-58 type carbapenemases and metal- -lactamase play significant roles in *A. baumannii* against carbapenems (Peleg et al., 2008; Walther-Rasmussen and Hoiby, 2006; Walsh, et al., 2005). Meanwhile, AdeABC and AdeIJK, members of the resistance–nodulation-cell division family (RND) efflux pump, have been reported in *A. baumannii* (Wieczorek et al., 2008; Magnet et al., 2001; Damier-Piolle et al., 2008).

In this study, all the 50 strains were divided into 5 gene types according to the REP-PCR. As reported previously (Huang et al., 2008), most of the isolates were belonging to the same type, containing the 22 imipenem-resistant isolates, 9 of Group B and 8 of C. In accordance with previous findings (Zhou et al., 2007), bla_{OXA-23} was existed in all the 22 carbapenems-resistant *A. baumannii* isolates in this study. These isolates showed identical genetical profile and antibiotic pattern. bla_{OXA-24}, bla_{OXA-58} and IPM, VIM, SIM-1 were undetectable in the all the 50 *A. baumannii* strains. The genes encoding the AdeABC efflux pump are located on the bacterial chromosome containing adeA, adeB and adeC. adeB captures its substrates either from within the phospholipids bilayer of the inner membrane or the cytoplasm in *A. baumannii* while adeA and adeC act as assistance (Magnet et al., 2001; Marchand et al., 2004). For all the *A. baumannii* strains studied here, adeB with the positive rate of 100%, not only in the 22 carbapenems-resistant *A. baumannii* isolates but also in the 28 carbapenems-sensitive ones. Sequencing was performed to confirm the gene. The high positive rate strongly indicate that AdeABC efflux pump is ubiquitous exists in A. baumannii, acting as a cryptic (Marchand et al., 2004) or even an intrinsic gene as $bla_{OXA-51-like}$. Given the fact that AdeABC efflux pump exists both in carbapenem-resistant and sensitive strains leave us the idea that it may not be an important contributor to carbapenems resistance.

Advanced study was carried out on the expression of adeB by Real-Time RT-PCR as result. The adeB mRNA of the 8 Group B isolates expressed steadily and identically with the C.V of 2.38% relative to their respectively 16sRNA. Compared with Group B, decreased expression was statistically significant ($P < 0.05$) both in Group A and C. The only exceptional was A2, giving 2.2 fold higher. It's in accordant to the phenotype analysis. This gives the further evidence that the AdeABC efflux pump act poor against carbapenem. Another study carried out by Simona Bratu and his colleagues have already supported the idea that the expression of adeB did not correlate with meropenem resistance (Bratu et al., 2008).

Table 2. Relative RNA expression level and efflux pump phenotype

Isolates	Relative expression	Inductive expression		phenotype
		IMP Pressure	MEM Pressure	
A1	0.2331	9.1	0.96	Negative
A2	1.386	0.22	0.41	Positive
A3	0.3969	4.9	0.5	Negative
A4	0.189	0.88	0.92	Negative
A5	0.4725	1.04	0.88	Negative
A6	0.5733	0.90	0.82	Negative
A7	0.693	1.02	0.95	Negative
A8	0.5985	0.91	0.89	Negative
B1	0.6489	N	N	N
B2	0.6363	N	N	N
B3	0.6111	N	N	N
B4	0.6237	N	N	N
B5	0.6048	N	N	N
B6	0.6615	N	N	N
B7	0.6174	N	N	N
B8	0.6363	N	N	N
C1	0.063	N	N	N
C2	0.1008	N	N	N
C3	0.1323	N	N	N
C4	0.015	N	N	N
C5	0.2205	N	N	N
C6	0.2646	N	N	N
C7	0.1386	N	N	N
C8	0.2331	N	N	N

Relative adeB mRNA expression to its own 16sRNA expression level. Relative expression level under the pressure of IMP and MEM to their isogenic counterparts growing on antibiotic-free M-H agar. N: Test has not been carried out.

The expression of the efflux pump has two types: inductive or constitutive, depending on whether induced by the substrate or independent of the environment (Wieczorek et al., 2008; Roberts et al., 1996). Thus, inductive effect of adeB mRNA expression was studied between strains grew under the pressure of imipenem and meropenem and their freely grew isogenic counter-parts on M-H agar. Increased expression of adeB was observed only in A1 and A3 under the pressure of imipenem. They expressed 9.1 and 4.2 fold higher, respectively, while decreased expression appeared in all the eight isolates under the pressure of meropenem. Puzzlingly, the expression of adeB of A2 was even a little inhibited both by imipenem and meropenem, 0.22 and 0.41 fold decreased respectively. It's possible that A2 expressed constitutively and inhibited by CCCP, so that appeared with a positive phenotype. Although, the expression of A1 and A3 was inducible by imipenem, the failure of the phenotype detection may owe the high expression of bla_{OXA-23}. It's false negative in another word. As reported, adeB high expression isolates without the other carbapenemases background may turn out to be with a positive phenotype (Huang et al., 2008). Then, it's suggested that AdeABC efflux pump may have effect on the susceptibility of carbapenems in *A. baumannii* but far from been a primary.

The expression of the AdeABC efflux pump is stringently regulated by the AdeRS two-component system. Either variation in AdeR or AdeS resulted in up-regulated expression of AdeABC efflux pump, causing muti-drug resistance (Marchand et al., 2004) . The response regulator- and sensor kinase-encoding gene, adeR and adeS, were amplified and sequenced. No mutations in the two regulative genes were observed in A1, A2 and A3 blasted with *A. baumannii* BM4454. No meaningful mutation points were found in the adeB over expression isolates by Huang and his colleagues, either (Huang et al., 2008) . It is possible that promoters such as ISAba1 play a role in the expression of AdeABC efflux pump (Peleg et al., 2008).

Because of the co-existence of -lactamases and efflux systems, assessment of the contribution of AdeABC

efflux pump is admittedly a difficult task. Gene knockout and restoration studies are still required to determine their precise roles in further studies.

Integrated with the evidence of its ubiquity both in carbapenem-resistant and sensitive strains, relatively low expression level and poor induction effect, we drawn the conclusion that AdeABC efflux pump play a less important role in *A. baumannii* against carbapenenms.

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