Original Article

Rabbit as a reservoir of multidrug-resistant *Acinetobacter baumannii* expressing the Ade multidrug efflux pumps

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Abstract

Acinetobacter baumannii has emerged as a nosocomial pathogen and considered as a major cause of hospital acquired infections. While *A. baumannii* in humans have been extensively studied in various situations, the study of *A. baumannii* in companion animals is still limited. The aim of this study was to examine the presence and antimicrobial resistance profile of *A. baumannii* isolates from companion rabbits. A total of 17 nasal swab from rabbit carcasses were obtained. *A. baumannii* was detected in 23.53% (4/17) of the samples and all the isolates exhibited resistance to chloramphenicol, erythromycin, spectinomycin and trimethoprim. Fifty percent of the isolates (2/4) were multidrug resistance (MDR) *A. baumannii* (ABR601 and ABR604) and produced all the Ade multidrug efflux tested including AdeABC, AdeFGH and AdeIJK. The other 2 isolates, including ABR602 and ABR603, demonstrated different expression profile of the Ade systems i.e. AdeIJK and AdeFGH-AdeIJK, respectively. Of all the isolates, ABR604 and ABR601 were resistant to 12 and 10 out of the 15 antimicrobials tested, respectively. This study indicates the presence of MDR *A. baumannii* in pets other than dogs and cats and highlights the possible role of these animals as reserviors for the spread of MDR *A. baumannii* to humans, animals and environment.

Keywords: *Acinetobacter baumannii*, Animal, Multidrug efflux pump, Multidrug resistance, Rabbit

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Introduction

Acinetobacter baumannii is a Gram-negative opportunistic pathogens that are frequently involved in nosocomial outbreaks and severely complicate sickness in patients (Kalil *et al*., 2016). This organism widely distributes in environment and is often associated with wound, urinary tract and respiratory tract infections in animals (Cisneros and Rodriguez-Bano, 2002). Recently, *A. baumannii* infections in companion animals including dogs, cats and horses have been increasingly reported (Abbott *et al*., 2005; Zordan *et al*., 2011). As seen in human medicine, the increasing emergence of multidrug resistance (MDR) *A. baumannii* in animals has raised a particular concern for the possible clonal transmission of these bacteria between humans and animals (Endimiani *et al*., 2011; Smet *et al*., 2012). Currently, there is an upward trend of bacterial pathogens (including *A. baumannii*) observed in exotic pets and the possible role of these animals as reservoirs for spreading the pathogens according to One Health concept has been suggested (Chomel *et al*., 2007). Unlike other companion animals, report of *A. baumannii* isolated from rabbit still limited.

One of the most important mechanisms underlying multidrug resistance in *A. baumannii* is overexpression of Ade multidrug efflux systems (Coyne *et al*., 2011). Three Ade Multidrug efflux pumps, including AdeABC, AdeFGH and AdeIJK, which are in the resistance-nodulation cell division (RND) family have been characterized and reported as a major mechanism responsible for development and spread of MDR *A. baumannii* (Leus *et al*., 2018). These efflux pumps have been frequently shown as the major contributors to high-level resistance to chloramphenicol, fluoroquinolones, tetracyclines and trimethoprim, however, some other antimicrobials have been recognized as specific substrates for particular Ade efflux pump specifically (e.g. AdeABC for aminoglycosides) (Magnet *et al*., 2001; Damier-Piolle *et al*., 2008; Coyne *et al*., 2010).

Up to date, *A. baumannii* infections in companion animals have been progressively concerned and increasingly reported (Wareth *et al*., 2019). The information of *A. baumannii* in rabbits has not been reported so far. Therefore, this study aimed to examine antimicrobial susceptibility and expression profile of four *A. baumannii* isolates obtained from rabbit carcasses. The data obtained could provide more sophisticated detail of *A. baumannii* in companion animals.

Materials and Methods

Bacterial isolates : The *A. baumannii* isolates in this study were obtained from nasal swab from rabbit carcasses submitted for pathological examination during September, 2016 to September, 2017 at Pathology Unit, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The organism was isolated and identified by using CHROMagar™ Acinetobacter medium (CHROMagar, Paris, France) followed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Dijkshoorn *et al*., 1998). All the isolates were examined of clonality and confirmed to be non-repetetive strains (data not shown) by using repetitive sequence based-PCR (rep-PCR) (Amonsin *et al*., 2003). All the bacterial cultures were grown on Luria Bertani (LB) agar or broth (Difco, BD Diagnostic Systems, MD, USA) and incubated at 37°C in aerobic conditions.

Antimicrobial susceptibility testing: Minimal inhibitory concentrations (MICs) of 15 clinically important antimicrobial agents were determined using two-fold agar dilution method (CLSI, 2015). The antimicrobial agents (with clinical breakpoints in parenthesis) included amikacin (64 µg/ml), aztreonam (32 µg/ml), carbenicillin (64 µg/ml), ceftazidime (32 µg/ml), chloramphenicol (32 µg/ml), ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), gentamicin (8 µg/ml), kanamycin (64 µg/ml), neomycin (32 µg/ml), piperacillin (128 µg/ml), spectinomycin (128 µg/ml), streptomycin (128 µg/ml), tetracycline (16 µg/ml), trimethoprim (4 µg/ml). All the antimicrobial agents were purchased from Sigma-Aldrich® (MO, USA), *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *Acinetobacter baumannii* ATCC19606 were used as control strains.

RNA extraction and cDNA synthesis: Total RNA was extracted using Total RNA Extraction Kit Mini (RBCBioscience, New Taipei City, Taiwan) in accordance with manufacturer's instructions. The extracted RNA concentration was determined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and treated with DNase I (Thermo Fisher Scientific, MA, USA) to remove remaining DNA. cDNA synthesization was performed using ImProm-II™ Reverse Transcriptase (Promega, WI, USA). The reverse transcription reaction was maded in a final volume 20 µl reaction. Each 5 µl reaction comprising 100 ng/µl DNase treated RNA and 1 µl of 10 µM reverse primer of each gene (AdeB_RV_P6, AdeG_Rv_P8, AdeJ_Rv_P10) was prepared and incubated at 70°C for 5 min and quick-chilled on ice for 5 min. The reverse PCR reaction consisted of 4 µl of ImProm-II™ 5X Reaction Buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix (10 mM each dNTP), 7 µl of nuclease-free water and 1 µl of ImProm-IITM reverse transcriptase. The reverse transcription steps included annealing at 25°C for 5 min, extension at 45°C for 45 min and heat inactivation at 70°C for 15 min. The synthesized cDNA was stored at -20°C until used.

Reverse transcription polymerase chain reaction (RT-PCR): cDNA of each *A. baumannii* isolate was used as DNA template in RT-PCR for detection of three clinically-important Ade efflux pumps (AdeABC, AdeFGH, AdeIJK). All the reactions were conducted in a 50 µl reaction mixture containing 25 µl of 2x KAPA Taq ReadyMix (Kapa Biosystems, MA, USA), 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 2 µl of the synthesized cDNA (25 ng/µl), 2.5 µl of 10 µM of each primer (Table 1) and nuclease-free water added to 50 µl. The cycle conditions were performed as follows, a cycle of 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 61-65°C for 45 s, 72°C for 1 min and a cycle of 72°C for 5 min. The RT-PCR products were analyzed on 1.5% agarose gel electrophoresis and

visualized by Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA). The *A. baumannii* ATCC19606 was used as reference control strain.

PCR amplification and DNA Sequencing: All the PCR amplifications were performed using KAPA Taq ReadyMix (Kapa Biosystems, MA, USA) according to the manufacturer's instructions. The PCR products from either RT-PCR and conventional PCR were purified by using Nucleospin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and submitted to First BASE Laboratories (Selangor, Malaysia) for nucleotide sequencing. Whole gene sequences obtained were analyzed by comparison to the sequence of *A. baumannii* reference ATCC19606 available in GenBank® database.

Table 1 Primer used in this study

Primer name	Sequence (5′ to 3′)	Target gene	Amplicon size (bp)	Reference
AdeB_Fw_P5	CCCTAATCAAGGACGTATGC	*adeB*	153	Pagdepanichkit *et al.* (2016)
AdeB_Rv_P6	GGTGCCTTATTCCATTGTGG			
AdeG_Fw_P7	TTATCAGGTCCGTGCACAAG	*adeG*	245	Pagdepanichkit *et al.* (2016)
AdeG_Rv_P8	GTGCAGCAATACGTTCAACC			
AdeJ_Fw_P9	GCCGTATGATGCCTGAAGAC	*adeJ*	346	Pagdepanichkit *et al.* (2016)
AdeJ_Rv_P10	GCAGCCAAGCAAAGGAATAC			

Results

Source of A. baumannii isolates in rabbit carcasses: From all the samples from 17 rabbits, four *A. baumannii* (23.53%) were obtained (Table 2). All the rabbits were mixed breed. Three of them were over 2 years of age and the others was 1.5 months old. Medical record showed that two rabbits (2 and 7 years old) were treated with enrofloxacin and marbofloxacin (data not shown). Detail of antibiotic treatment of the other rabbits was not disclosed.

Antimicrobial susceptibility tesing: In this study, MDR was defined as being resistant at least 6 antimicrobial drugs (Gu *et al*., 2007; Poonsuk *et al*., 2012). All the *A. baumannii* isolates were resistant to chloramphenicol, erythromycin, spectinomycin and trimethoprim. Two isolates, including ABR601 and ABR604, were resistant to 10 and 12 antimicrobials respectively (Table 2) and defined as MDR *A. baumannii*. The other two isolates (ABR602, ABR603) were resistant to 4 antimicrobials included chloramphenicol, erythromycin, spectinomycin and trimethoprim.

Expression profile of the clinically-important Ade multidrug efflux pumps: The expression profile of the Ade efflux pumps of all clinical isolates is shown in Table 3. The MDR *A. baumannii* (ABR601, ABR604) produced all the Ade efflux pumps tested including AdeABC, AdeFGH and AdeIJK. ABR602 exhibiting the lowest MICs for amikacin (4 µg/ml) and trimethoprim (8 µg/ml) expressed only AdeABC. ABR603 resistant to chloramphenicol and spectinomycin simultaneously expressed AdeFGH and AdeIJK.

Table 2 Antimicrobial susceptibility in the *A. baumannii* isolates obtained from rabbit.

Clinical isolates	MIC (µg/ml)[a]														
	AMK	ATM	CAR	TAZ	CHL	CIP	ERY	GEN	KAN	NEO	PIP	SPE	STR	TET	TMP
ABR601	16	8	8	4	**256**	**64**	**32**	**32**	**512**	**128**	≤2	**>1024**	**1024**	**>256**	**64**
ABR602	4	16	16	4	**128**	≤0.06	16	2	4	8	4	**128**	64	1	**8**
ABR603	8	16	16	4	**256**	≤0.06	16	2	4	8	4	**128**	64	1	**16**
ABR604	**128**	**32**	**64**	16	**512**	**64**	**32**	**32**	**>512**	16	64	**128**	**1024**	**>256**	**128**

[a] , values in boldface indicate MICs that are resistant to each antimicrobials tested.
Abbreviations: AMK, amikacin; ATM, aztreonam; CAR, carbenicillin; TAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; PIP, piperacillin; SPE, spectinomycin; STR, streptomycin; TET, tetracycline; TMP, trimethoprim.

Table 3 Expression of the Ade efflux pumps determined by RT-PCR in the *A. baumannii* isolates.

Clinical isolates	Ade efflux pumps expressed
ABR601	AdeABC, AdeFGH, AdeIJK
ABR602	AdeIJK
ABR603	AdeFGH, AdeIJK
ABR604	AdeABC, AdeFGH, AdeIJK

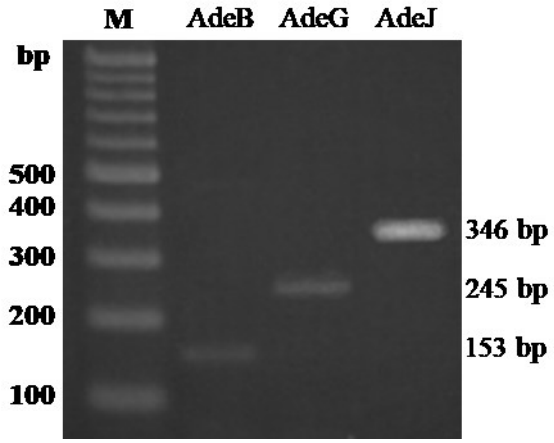

Figure 1 PCR amplicons of Ade multidrug efflux systems. Lane M, 100 bp molecular weight marker; Lane 2, *adeB ;* Lane 3, *adeG* and Lane 4, *adeJ*, respectively.

Discussion

A. baumannii has been recognized as a potential veterinary pathogen and MDR *A. baumannii* isolates have been increasingly reported worldwide (Wareth *et al*., 2019). In this study, the percentage of *A. baumannii* isolated from rabbits was 23.53%, which was approximately 3-4 times higher than the previous studies (6.5-7%) conducted in other animals (Black *et al*., 2009; Belmonte *et al*., 2014). The observation may be also affected by the limited number of the samples obtained in this study. Since rabbits have gained more popularity as pets, this raises the particular concern of their role as reservoirs for MDR *A. baumannii*. ABR604 was isolated from a 7 years old rabbit that was dead due to chronic infection of respiratory tract. *A. baumannii* might be an etiologic cause of death in this animal as the pathogen usually complicates infection of respiratory tract in animals (Francey *et al*., 2000). ABR601and ABR604 exhibiting multidrug resistance phenotype were isolated from 2 rabbits that were exposed to fluoroquinolones before death and expressed all the Ade efflux pumps tested. Induction of Ade efflux pump expression by exposure to fluoroquinolones has been demonstrated and the important contribution of fluoroquinolones exposure in multidrug resistance phenotype of *A. baumannii* has been suggested (Zhang *et al*., 2017). The CDC annual report showed that up to 65% of all the human isolates were MDR *A. baumannii* (Tacconelli *et al*., 2014). Due to limited number of samples and *A. baumannii* isolates in this study, the comparison is not possible. Therefore, the study in a larger number of *A. baumannii* isolates is required.

A previous study showed that only AdeABC was responsible for the efflux of aminoglycosides in *A. baumannii* (Magnet *et al*., 2001). Expression of all 3 Ade efflux pumps has been previously shown in MDR *A. baumannii* human clinical isolates with high antimicrobial resistance level (Yoon *et al*., 2013). However, it was shown that expression of these Ade efflux pump did not always result in high level of resistance to antimicrobials. Coexistence of other resistance mechanisms that were attributed to such high resistance level was suggested (D'Souza *et al*., 2019). Resistance to most aminoglycosides tested including gentamicin, kanamycin, spectinomycin and streptomycin in ABR601 and ABR604 was possibly attributed (in part) to expression of AdeABC. All the isolates in this study expressed AdeIJK. This is not surprising because the efflux system is constitutively expressed at low level and contributed to intrinsic resistance to various antimicrobials (e.g. chrolamphenicol, fluoroquinolones, tetracyclines) (Rosenfeld *et al*., 2012). Further investigation to determine the actual contribution of the Ade systems expressed in the present study (e.g. gene replacement) is recommended.

Based on our knowledge, this is the first report of MDR *A. baumannii* in rabbits in Thailand. Despite the limited number of the *A. baumannii* isolates, the results point out the simultaneous expression of the Ade multidrug efflux pumps in the animal isolates. Furthur studies are warranted to explore the contribution of the Ade systems to the MDR phenotype. The study to discover efflux pump inhibitors (EPIs) as a promising strategy to fight against MDR *A. baumannii* in animals is suggested.

In conclusion, this study highlights the presence of MDR *A. baumannii* isolates exhibiting expression of AdeABC, AdeFGH and AdeFGH in rabbit carcasses. Expression of all Ade efflux pumps in the rabbit clinical isolates might be responsible for antimicrobial resistance phenotype of these isolates and further studies are warranted to elucidate the role of the Ade efflux systems. Molecular characterization of the Ade efflux systems and other resistance determinants of *A. baumannii* animal isolates is suggested, as seen in other opportunistic pathogens such as *Pseudomonas* spp. Besides, the study of genetic relatedness of the *A. baumannii* isolates from humans and animals is suggested to investigate clonal spread.

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