

Presence of the Ade multidrug systems and response to triclosan exposure in *Acinetobacter baumannii* from animal origins

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Abstract

Thirty *Acinetobacter baumannii* from animal origins were obtained from 210 animal carcasses submitted for post-mortem examination, including dogs (n=112), cats (n=45), rabbits (n=17), birds (n=15), pigs (n=7) and exotic animals (n=14). All the isolates were evaluated for antimicrobial susceptibility with the absence and presence of efflux inhibitors, i.e., reserpine and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and expression profile of important six multidrug efflux systems (i.e., AdeABC, AdeDE, AdeFGH, AdeIJK, AbeM and AmvA). Fifteen isolates expressing different Ade multidrug efflux pumps were selected to determine transcription levels and regulatory mutations. A continuous *in vitro* exposure experiment with benzalkonium chloride, chlorhexidine, and triclosan was performed in the isolates susceptible to most antibiotics and biocides tested (n=12). The results showed that 70% of the isolates (n=21) were multidrug-resistant, and all expressed AdeIJK. The inhibitory effect of reserpine and CCCP suggested that active efflux pumps using a different type of energy (i.e., ATP and proton-motif force) contribute to the reduced susceptibility to antimicrobials in the isolates. Exposure to triclosan resulted in a spontaneous-mutant derivative, ABJ302-1, exhibiting cross-resistance to 10 of 15 antibiotics tested and resulted in downregulated expression of AdeG and AdeJ in almost all the isolates, which may be affected by the stress response of triclosan.

Keywords: *Acinetobacter baumannii*, animals, cross-resistance, multidrug efflux systems, triclosan

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Introduction

Among the *Acinetobacter* genus, *A. baumannii* is the most clinically important species and is one of the leading causes of severe nosocomial infections. The pathogen is infamously known for its intrinsic resistance and the remarkable ability to acquire resistance to several antimicrobials rapidly after exposure (Singh *et al.*, 2013). Nosocomial infections linked to *A. baumannii* have been reported in veterinary clinics and should be highlighted for their clinical significance. (Zordan *et al.*, 2011; van der Kolk *et al.*, 2019). Multidrug-resistant *A. baumannii* (MDRAB) were isolated from many animal species (e.g., dogs, cats, and horses) and involved in infectious conditions in various organs such as respiratory, ophthalmic, urinary tract, and bloodstream infections (Endimiani *et al.*, 2011). Moreover, this organism can also be considered a zoonotic pathogen, possibly transmitted from humans to animals (Nocera *et al.*, 2020). The emergence of carbapenemase-producing *A. baumannii* has been reported in Thailand, and the spread of MDRAB in veterinary settings is expected to rise (Leelapsawas *et al.*, 2022). However, the knowledge of MDRAB of animal origin is still limited.

Multidrug efflux systems in the resistance-nodulation cell division (RND) family, including AdeABC, AdeDE, AdeIJK, and AdeFGH, have emerged as a major mechanism underlying MDR phenotype in *A. baumannii* (Leus *et al.*, 2018). AdeABC is tightly regulated by the AdeRS-two component system, and its substrates include aminoglycosides, fluoroquinolones, erythromycin, tetracycline, trimethoprim, and chloramphenicol (Marchand *et al.*, 2004). AdeDE was mainly found in *Acinetobacter* genomic DNA group 3 (GDG3), but it was recently observed in *A. baumannii* clinical isolates and mediated resistance to amikacin, tetracycline, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, and rifampicin (Hou *et al.*, 2012). AdeIJK is constitutively expressed and is under the regulation of *adeN* (Rosenfeld *et al.*, 2012). Its substrates include β -lactams, chloramphenicol, tetracycline, erythromycin, lincosamide, fluoroquinolones, fusidic acid, rifampicin, trimethoprim, novobiocin and clindamycin (Rosenfeld *et al.*, 2012). AdeFGH effluxes chloramphenicol, clindamycin, fluoroquinolones, sulfamethoxazole, tetracycline, tigecycline, and trimethoprim, and its overexpression was identified in the laboratory strains lacking AdeABC and with *adeL* mutation (Coyne *et al.*, 2010).

The evolutionary adaptation of bacteria to nonantibiotic selective pressure leading to increased antibiotic resistance has been well acknowledged. It was demonstrated that exposure to biocides could promote cross-resistance to antibiotics in clinically important *Pseudomonas aeruginosa*, and the RND-type efflux pumps contributed to such cross-resistance (Chuanchuen *et al.*, 2001). In *A. baumannii*, AbeM and AmvA of the MFS and MATE efflux transporters, respectively, were shown to extrude disinfectants (e.g., benzalkonium chloride, chlorhexidine, triclosan) and multiple antibiotics (e.g. ciprofloxacin, erythromycin, gentamicin) simultaneously (Su *et al.*, 2005; Rajamohan *et al.*, 2010). These observations have placed

disinfectants used in home and healthcare settings as a contributor to cross-resistance to clinically important antibiotics, and thus, 19 antimicrobial ingredients, including triclosan, have been banned by US Food and Drug Administration (US FDA) (Halden *et al.*, 2017). Due to the limitation of knowledge on MDRAB in animals, we aimed to investigate the antimicrobial resistance profile, expression of the Ade multidrug efflux systems, and their contribution to cross-resistance between disinfectants and antibiotics in *A. baumannii* from animals.

Materials and Methods

Sample collection and bacterial isolation: Two hundred and ten nasal swabs were obtained from whole animal carcasses including dogs (n=112), cats (n=45), rabbits (n=17), pigs (n=5), hedgehogs (n=4), lizards (n=3), monkeys (n=2), hamsters (n=3), gatsby (n=1), and chinchilla (n=1) from September 2017-September 2018. The causes of death included respiratory infections and chronic diseases such as heart disease, cancer, and diabetes. All the carcasses were submitted to the Pathology Unit, Faculty of Veterinary Science, Chulalongkorn University, for necropsy. One hundred sixty-four carcasses originated from Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, and submitted to the unit as part of routine *pathological examination*. Twenty-one carcasses were from private animal hospitals. The other carcasses were obtained from bird farms (n=14), pig farms (n=5), national zoos (n=2), and animal owners (n=4). All carcasses were shipped overnight on ice in an insulated container by veterinarians, except those from the Small Animal Teaching Hospital of Chulalongkorn University. Upon their arrival at the Pathology Unit, all carcasses were stored at 4°C until necropsy. The samples were collected using sterile cotton swabs within 8 hours after the animal carcasses arrived.

Each swab sample was directly streaked on selective medium CHROMagar™ *Acinetobacter* medium (CHROMagar, Paris, France) and incubated at 37°C overnight. Three to five typical red colonies of *Acinetobacter* spp. were randomly picked from each positive sample and confirmed as *A. baumannii* by using amplified ribosomal DNA restriction analysis (ARDRA) (Dijkshoorn *et al.*, 1998). A single colony of *A. baumannii* from each positive sample was collected and stored at -80°C in 20% glycerol. *A. baumannii* ATCC 19606 was used as a control strain for ARDRA.

Antimicrobial susceptibility testing: Minimal inhibitory concentrations (MICs) of 15 antimicrobial drugs and three disinfectants (i.e., benzalkonium chloride (BZK), chlorhexidine (CHX), and triclosan (TCS)) were determined by using 2-fold agar dilution method according to Clinical and Laboratory Standards Institute (CLSI, 2015). Antimicrobial agents used and their clinical breakpoints (in parenthesis) were amikacin (AMK, 16 µg/ml), aztreonam (ATM, 32 µg/ml), carbenicillin (CAR, 64 µg/ml), ceftazidime (TAZ, 32 µg/ml), chloramphenicol (CHL, 32 µg/ml), ciprofloxacin (CIP, 4 µg/ml), erythromycin (ERY, 8 µg/ml), gentamicin (GEN, 8 µg/ml), kanamycin

(KAN, 64 µg/ml), neomycin (NEO, 32 µg/ml), piperacillin (PIP, 128 µg/ml), spectinomycin (SPE, 128 µg/ml), streptomycin (STR, 128 µg/ml), tetracycline (TET, 16 µg/ml), trimethoprim (TMP, 4 µg/ml). All antimicrobial drugs and disinfectants were purchased from Sigma-Aldrich® (St. Louis, MO, USA). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were served as reference strains.

The activity of efflux pump inhibitors (EPIs): MICs of 15 antimicrobial agents were determined in the presence and absence of 25 µg/ml reserpine (Sigma-Aldrich) or 5 µg/ml carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) (Sigma-Aldrich) in all the isolates. At least a 4-fold decrease in the MIC values after the addition of reserpine or CCCP was considered significantly different.

Table 1 Primer used in this study.

Primer name	Sequence (5' to 3')	Gene	Tm (°C)	Reference
Universalprimerup	TGGCTCAGATTGAACGCTGGCGGC	16S rRNA	50	Vaneechoutte <i>et al.</i> , 1995
Universalprimerdown	TACCTTGTTACGACTTCACCCCA			
AdeB_Fw_P5	CCCTAATCAAGGACGTATGC	<i>adeB</i>	61	Pagdepanichkit <i>et al.</i> , 2016
AdeB_RV_P6	GGTGCCTTATTCCATTGTGG			
AdeG_Fw_P7	TTATCAGGTCCGTGCACAAG	<i>adeG</i>	61	Pagdepanichkit <i>et al.</i> , 2016
AdeG_RV_P8	GTGCAGCAATACGTTCAACC			
AdeJ_Fw_P9	GCCGTATGATGCCTGAAGAC	<i>adeJ</i>	62	Pagdepanichkit <i>et al.</i> , 2016
AdeJ_Rv_P10	GCAGCCAAGCAAAGGAATAC			
AdeE_Fw_P11	CAGGGACGTGTTAAGCGAGT	<i>adeE</i>	65	Pagdepanichkit <i>et al.</i> , 2016
AdeE_Rv_P12	CCAGACTTGCAGCCACTACA			
AdeL_Fw_P19	ATTTCGAACTTACTCATATGCTGA	<i>adeL</i>	53	Pagdepanichkit <i>et al.</i> , 2016
AdeL_Rv_P20	GGAATGGACGGAGCATAAAA			
AdeN_Fw_P21	AAGCAGTGTTAGCCGTCGTT	<i>adeN</i>	58	Pagdepanichkit <i>et al.</i> , 2016
AdeN_Rv_P22	GTIGTTGGCTGGGTGGAAGT			
AmvA_Fw_P23	AATCCTGACAATTATCGTCCTCA	<i>amvA</i>	60	This study
AmvA_Rv_P24	TCTTCGAAAACTAAACCAGACA			
AbeM_Fw_P25	TGTCGAATGTCACGTCGTTT	<i>abeM</i>	59	This study
AbeM_Rv_P26	CGTTTGGTATTCAAATAGAGTCG			
rpsLrealtimeup	CGGCACTGCGTAAGGTATGC	<i>rpsL</i>	62	Chuanchuen <i>et al.</i> , 2008
rpsLrealtimedown	CCCGGAAGGTCCTTTACACG			
ERIC IR	ATGTAAGCTCCTGGGGATTACAC	Rep-PCR	45	Amonsin <i>et al.</i> , 2003
ERIC II	AAGTAAGTGACTGGGGTGAGCCG			

Reverse transcription (RT)-PCR: Total RNA was extracted from late log phase culture using GeneJET RNA Purification Kit (Thermo Fisher Scientific, MA, USA) as described by the manufacturer. The RNA concentration was determined by using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). The RNA samples were treated with DNaseI (Thermo Fisher Scientific, MA, USA) and used for cDNA synthesis using ImProm-II™ Reverse Transcriptase (Promega, WI, USA). One µg of total RNA was mixed with 1 pmol of the reverse primer. Reverse transcription was carried out at 25°C for 5 min, 45°C for 45 min, and 70°C for 15 min. Then, amplifications of *adeB*, *adeG*, *adeJ*, and *adeE* were conducted using conventional PCR with specific pairs of primers in a total of 20 µl reaction volume in TPersonal combi thermocycler (Biometra, Germany). Thermocycle condition includes a cycle of 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 61-65°C for 45 s and 72°C for 1 min, and final extension at 72°C for 5 min.

Polymerase chain reaction (PCR) and nucleotide sequencing: All PCR primers used in this study are listed in Table 1. Amplification of *adeL* and *adeN* was performed in 25 µl reaction mixture containing 12.5 µl of KAPA TaqReadyMix PCR Kit (KAPABIOSYSTEMS, MA, USA), 0.5 µM of each forward and reverse primer, 25 ng of DNA template and 10 µl of nuclease-free water. Amplicons were purified using Nucleospin® PCR clean-up Gel extraction (Macherey-Nagel Inc. PA, USA). Amplicons were sequenced in both directions at First BASE Laboratories (Selangor, Malaysia). Nucleotide sequences obtained were compared to those of *A. baumannii* ATCC 19606 in the GenBank® database (<http://www.ncbi.nlm.nih.gov/genbank/>).

Quantitative reverse transcriptase (qRT)-PCR: The expression level of the RND transporters, including *adeB*, *adeG*, and *adeJ*, was determined using real-time qRT-PCR. The cDNA samples were synthesized as described above and diluted to obtain a cDNA template at the concentration of 2.5 ng/µl. The total 20 µl qRT-PCR reaction mixture comprised of 10 µl of KAPA SYBR® FAST qPCR Master Mix Universal (KAPABIOSYSTEMS, MA, USA), 200 nM of each forward and reverse primer, 12.5 ng of diluted cDNA template and 3 µl of nuclease-free water. qRT-PCR was performed in Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia). The expression level of each gene was calculated as a fold change ratio compared with a standard curve constructed against known DNA concentrated of *A. baumannii* ATCC 19606 genomic DNA. Each experiment was carried out in triplicate, and the *rpsL* gene was used as an internal control. The transcription level at least 3-fold higher than that in ATCC19606 was considered significant (Islam *et al.*, 2004).

In vitro exposure assay: Fifteen *E. coli* isolates with low MICs of the biocides tested (MIC range 16-64 µg/ml for BZK; 8-64 µg/ml for CHX; and 0.5-8 µg/ml for TCS) and susceptible to most antibiotics were selected. *In vitro* exposure to gradually increasing concentrations of BZK, CHX, and TCS was performed as previously described with some modifications (Gradel *et al.*, 2005). Briefly, 100 µl of the 37°C-overnight grown inoculum was transferred to 4 ml fresh LB broth containing BZK, CHX, or TCS at a concentration of 0.25-fold MIC of each disinfectant and incubated at 37°C for 18-20 h. If bacterial growth was observed, 100-200 µl of the inoculum was spread onto LB agar without disinfectants to check the purity and the existence of bacteria. The procedure was repeated daily in LB broth with 1.5-fold increasing concentrations of BZK, CHX, or TCS until no growth was observed. Spontaneous mutant derivatives from the final passage that grew in LB broth with ≥ 4 -fold MICs of their isogenic parental strains were collected. The isolates were grown by streaking on LB agar without disinfectant daily for up to 20 consecutive days. The continuation of spontaneous mutant derivatives and parental strains was confirmed by repetitive sequence based-PCR (rep-PCR) using the primer pairs ERIC IR and ERIC II (Amonsin *et al.*, 2003).

All the mutant derivatives obtained and their parental strains were determined for transcription of *adeB*, *adeG*, *adeJ*, *amvA*, and *abeM* by RT-PCR, and then, the transcription level of the RND transporters was examined using qRT-PCR. Detection of regulatory mutations of *adeN* and *adeL* genes was performed by using conventional PCR and nucleotide sequencing as described above.

Result

***Acinetobacter baumannii* from animal origins:** Of all 210 samples, the typical red colonies of *Acinetobacter* were observed in 59 samples, of which 30 samples were confirmed to be positive for *A. baumannii* (14.29%). These included the samples from dogs (n=7, 15.18%), cats (n=8, 17.78%), rabbits (n=4, 23.53%), and pigs (n=1, 14.29%) (Table 2). The other *Acinetobacter* species identified included *A. haemolyticus* (n=2), *Acinetobacter* genomic species 3 (n=2), *Acinetobacter* Gen. sp. *Sensu* Bouvet and Jeanjean (BJ) (n=1) and *A. lwoffii* (n=1). Thirty *A. baumannii* isolates collected for further experiments originated from dogs (56.67%), cats (26.67%), rabbits (13.33%), and pigs (3.33%).

Antimicrobial susceptibilities: All the *A. baumannii* isolates (n=30) were tested for their antimicrobial susceptibilities and exhibited resistance to CHL (100%) (Table 3). Resistance rates to TMP, ERY, SPE, and CIP were 97%, 93%, 83%, and 70%, respectively. Resistance rates to ATM and TET were 63% of each, while those for STR and GEN were 53% of each. Seventy percent of the isolates exhibited multidrug resistance phenotype (MDR, being resistant to at least 6 different antimicrobial drugs) (Gu *et al.*, 2007; Poonsuk *et al.*, 2012). The most common resistance pattern was CHL-ERY-SPE-TMP (13.3%), including the isolates from a dog (n=1), a cat (n=2), and a rabbit (n=1).

Based on clinical breakpoints, the effect of reserpine and CCCP on resistance rates varied. Overall, the presence of reserpine and CCCP resulted in a 3-26% reduction of resistance rates to AMK, ATM, CAR, TAZ, ERY, NEO, and SPE (Table 3).

The addition of reserpine reduced resistance rates to AMK, ATM, CAR, TAZ, ERY, NEO, SPE, and TET (3-26%), of which the greatest reduction was observed for ATM (from the resistance rate of 63% to 37%). The addition of reserpine resulted in the significant reduction of MICs for ERY, SPE, and TMP (3.3% of each), CAR and TAZ (6.7% of each), and NEO and TET (13% of each).

The presence of CCCP decreased the resistance rates to AMK, ATM, CAR, TAZ, CHL, ERY, NEO, and SPE (3-26%). The highest reduction of resistance rate was observed for SPE (from the resistance rate of 83% to 57%). At least 4-fold reduction of MICs was observed for NEO (40%), CHL (13%), ERY, STR, TET and TMP (10% each), AMK and PIP (6.7% each), CAR, TAZ, GEN, KAN and SPE (3.3% each).

Distribution of RND multidrug efflux pumps: All the *A. baumannii* isolates (n=30) were examined for the expression of *adeB*, *adeG*, *adeJ*, and *adeE* by RT-PCR. Transcription of *adeJ* was identified in all the isolates, while no *adeE* transcription was observed. The expression of AdeB and AdeG was detected in 53.33% and 90% of the isolates, respectively. Most isolates simultaneously expressed three Ade multidrug pump efflux pumps, AdeB-AdeG-AdeJ (53.33%). The other expression patterns observed were AdeJ (10%) and AdeG-AdeJ (36.7%).

Expression level of Ade multidrug efflux pumps and regulatory gene mutation: Fifteen isolates expressing different Ade multidrug efflux pumps were randomly selected (n=15) and examined for transcription level and regulatory mutation (Table 4). In comparison to *A. baumannii* ATCC 19606, none of the isolates overexpressed *adeB*. Overexpression of *adeG* was detected in 10 isolates (7.4 fold to 214.8 fold). Eleven isolates overexpressed *adeJ* (6.3 fold to 16.7 fold). Nine isolates (i.e. ABJ302, ABJ303, ABJ311, ABJ319, ABJ320, ABJ321, ABJ325, ABJ328 and ABJ329) overexpressed both *adeG* and *adeJ*, simultaneously. Interestingly, one of these isolates, ABJ302, was resistant to CHL only and carried amino acid change, Ile45-Val, in AdeL.

Ten isolates contained amino acid substitutions in AdeN, of which Met197-Thr was the most common (n=7). Four isolates (ABJ311, ABJ320, ABJ324 and ABJ331) carried same pattern amino acid replacements (Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr) in AdeN. Two AdeN-overexpressing isolates (ABJ321 and ABJ328) harbored Val44-dup. However, ABJ303 overexpressing 6.3 fold of *adeJ* did not carry any mutation in AdeN. ABJ316 and ABJ324, both resistant to ATM-CHL-CIP-ERY-SPE-STR-TET-TMP, did not overexpress any multidrug efflux pumps tested.

Characteristics of spontaneous triclosan-resistant mutant derivatives: Fifteen isolates that were susceptible to most antimicrobial agents tested and had low MICs for BZK, CHX, and TCS were selected for exposure to gradually increased concentrations of

BZK, CHX, and TCS. None of the isolates were able to grow in LB containing BZK and CHX at the concentration above 4-fold of MIC. In contrast, 12 isolates could grow in LB containing TCS at concentrations above 4-fold of their MICs (Table 5).

When considered resistance phenotypes, exposure to TCS resulted in increasing MICs (≥ 4 fold) of various antibiotics, including ATM, CAR, TAZ, CIP, ERY, NEO, PIP, SPE, STR, TET and TMP in 11 triclosan-resistant mutants, except ABJ311-1. None of the isolates developed higher MIC levels of CHL, GEN, KAN, and STR. All the triclosan-resistant isolates exhibited resistance to one to 10 antibiotics. ABJ302-1 developed resistance to all antibiotics tested, while its parent ABJ302 was resistant to only CHL. Five TCS-resistant mutant derivatives (i.e., ABJ316-1, ABJ317-1, ABJ324-1, ABJ328-1, and ABJ302-1) developed resistance to at least five antibiotics in comparison to their isogenic parents.

Based on RT-PCR, exposure to TCS did not affect the expression of *AmvA* in any isolates. At the same time, three isolates (i.e., ABJ311, ABJ324, and ABJ331)

expressed *AbeM* after exposure to TCS (i.e., ABJ311-1, ABJ324-1, and ABJ331-1). ABJ324-1 and ABJ331-1 developed resistance to ATM, CAR, CAZ, CIP, and ERY. ABJ324-1 is additionally resistant to PIP. In contrast, ABJ311-1 did not show resistance to any antibiotics tested.

The reduction of transcription level of *adeB*, *adeG*, and *adeJ* was found in almost all triclosan-resistant derivatives. The exception was observed for *adeJ* in ABJ324-1 (0.1 fold to 2.6 fold) and ABJ331-1 (2.4 fold to 3.3 fold).

Exposure to TCS resulted in regulatory mutations in *AdeN* of two triclosan-resistant isolates, i.e., amino acid change Ala32-Thr in ABJ303-1 and frameshift mutation Gly69-TyrfsTer28 in ABJ316-1. No regulatory mutation was observed in the other isolates. Amino acid substitution Ile145-Val in *AdeL* of ABJ302 (with 108.4 fold *AdeG* expression) was absent after exposure to TCS (ABJ302-1 with 3.7 fold *AdeG* expression). Despite the reduction of *AdeG* expression, ABJ302-1 developed resistance to 11 antibiotics.

Table 2 The presence of *A. baumannii* in the animal samples (n=210).

Species	No. of samples (n)	No. of <i>A. baumannii</i> positive samples (n, %)
Dog	112	17 (15.18)
Cat	45	8 (17.77)
Rabbit	17	4 (23.53)
Bird	15	0 (0)
Pig	7	1 (14.29)
Others	14	0 (0)
Total	210	30 (14.29)

^aOthers; hedgehog (n=4), lizard (n=3), monkey (n=2), hamster (n=3), Gatsby (n=1) and chinchilla (n=1)

Table 3 Antimicrobial susceptibility of *A. baumannii* animal isolates in the presence and absence of reserpine and CCCP (n=30).

Antimicrobial agent	None Resistance rate (%) ^a	+ RES		+ CCCP		MIC reduction range (fold) ^b		Rate of ≥4-fold MIC reduction (%) ^c	
		Resistance rate (%) ^a	% reduction	Resistance rate (%) ^a	% reduction	+ RES	+ CCCP	+ RES	+ CCCP
AMK	20	17	3	10	10	1-2	1-4	0	6.7
ATM	63	37	26	43	20	1-2	1-2	0	0
CAR	43	30	13	30	13	1-4	1-4	6.7	3.3
TAZ	23	20	3	20	3	1-4	1-4	6.7	3.3
CHL	100	100	0	97	3	1-2	1-8	0	13
CIP	70	70	0	70	0	1-2	1-2	0	0
ERY	93	90	3	87	6	1-4	1-4	3.3	10
GEN	53	53	0	53	0	1-2	1-4	0	3.3
KAN	30	30	0	30	0	1	1-4	0	3.3
NEO	17	7	10	7	10	1-4	1-8	13	40
PIP	23	23	0	23	0	1-2	1-4	0	6.7
SPE	83	60	23	57	26	1-4	1-4	3.3	3.3
STR	53	53	0	53	0	1-2	1-4	0	10
TET	63	53	10	63	0	1-128	1-4	13	10
TMP	97	97	0	97	0	1-4	1-8	3.3	10

^a None, no reserpine and CCCP added; + RES, reserpine added; + CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone added.
^b MIC reduction range as a result of the addition of RES or CCCP.
^c Percentage of 4-fold MIC reduction as a result of the addition of RES or CCCP.
Abbreviation: 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 4 Expression of the Ade efflux systems and mutations in the corresponding regulatory regions in *A. baumannii* animal clinical isolates (n=15).

Strain	Transcription level ^a			Regulatory mutation ^b		Antimicrobial resistance pattern
	<i>adeB</i>	<i>adeG</i>	<i>adeJ</i>	<i>AdeL</i>	<i>AdeN</i>	
19606	1	1	1	-	-	ATM-CHL-ERY-GEN-SPE-STR-TMP
ABJ302	-	108.4	11.6	Ile145-Val	Asn195-His, Met197-Thr	CHP
ABJ303	2.4	19.6	6.3	-	-	CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
ABJ308	-	-	6.6	-	Gln94-Arg	CHL-ERY-SPE-TMP
ABJ311	1.3	13.4	13.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
ABJ316	0.2	2.0	2.0	-	-	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP
ABJ317	-	0.1	8.0	-	ND	ATM-CHL-ERY-TMP
ABJ319	2.4	7.4	14.1	-	Pro16-Ser, Met186-Ile, Met197-Thr	CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
ABJ320	2.2	33.4	14.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
ABJ321	0.1	62.3	16.7	-	Val44dup	ATM-CAR-CHL-CIP-ERY-GEN-TET-TMP
ABJ323	-	-	0.1	-	ND	AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TMP
ABJ324	-	-	0.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP
ABJ325	-	42.0	6.8	-	ND	ATM-CHL-ERY-TMP
ABJ328	-	214.8	8.4	-	Met197-Arg	ATM-CHL-CIP-ERY-SPE-TMP
ABJ329	-	19.8	7.8	-	Val44dup	ATM-CHL-CIP-ERY-SPE-TET-TMP
ABJ331	2.2	10.3	2.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CAR-CHL-CIP-ERY-GEN-PIP-SPE-STR-TET-TMP

^a -, no expression.
^b -, no mutations found; ND, not detected.

Table 5 Characteristics of the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* animal clinical isolates.

Strain ^a	MIC (µg/ml) ^b										RT-PCR ^c		Transcription level ^d			Regulatory mutation ^e	
	TCS	ATM	CAR	TAZ	CIP	ERY	NEO	PIP	SPE	TET	TMP	AbeM	adeB	adeG	adeJ	Adel	AdelN
ABJ302	1	8	8	0.5	0.03	0.5	1	4	32	0.5	1	+	-	108.4	11.6	Ile145-Val	Asn195-His, Met197-Thr
ABJ302-1	16	128	128	16	1	128	4	64	128	2	16	+	-	3.7	3.0	-	Asn195-His, Met197-Thr
ABJ303	4	16	16	4	32	16	8	2	1024	128	4	+	2.4	19.6	6.3	-	-
ABJ303-1	32	32	64	16	64	64	8	32	1024	128	8	+	0.1	-	1.6	-	Ala32-Thr
ABJ311	2	32	16	8	64	32	8	16	512	>256	64	-	1.3	13.4	13.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ311-1	64	32	32	16	64	64	8	32	512	>256	64	+	0.1	0.6	2.6	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ316	2	32	16	4	16	16	8	2	128	>256	32	-	0.2	2.0	2.0	-	-
ABJ316-1	32	256	1024	32	> 64	64	8	256	128	>256	32	-	-	-	0.2	-	Gly69-TyrfsTer28
ABJ317	0.5	32	16	4	0.06	16	8	8	64	1	32	-	-	0.1	8.0	-	ND
ABJ317-1	32	256	128	32	0.25	16	8	64	64	2	32	-	-	-	2.9	-	ND
ABJ319	8	16	>1024	8	64	>128	8	16	1024	>256	64	+	2.4	7.4	14.1	-	Pro16-Ser, Met186-Ile, Met197-Thr
ABJ319-1	64	256	>1024	256	64	>128	8	512	1024	>256	64	+	0.1	0.8	2.7	-	Pro16-Ser, Met186-Ile, Met197-Thr
ABJ320	1	32	64	8	64	32	8	16	512	>256	64	+	2.2	33.4	14.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ320-1	256	128	64	32	64	128	8	64	1024	>256	64	+	0.1	0.4	3.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ321	2	32	1024	16	64	32	8	64	64	>256	64	+	0.1	62.3	16.7	-	Val44dup
ABJ321-1	256	256	1024	32	64	128	8	256	64	>256	64	+	-	1.0	3.3	-	Val44dup
ABJ323	0.5	>256	>1024	>256	1	16	64	>512	>1024	0.5	>128	-	-	-	0.1	-	ND
ABJ323-1	32	>256	>1024	>256	32	64	64	>512	>1024	0.5	>128	-	-	0.1	-	-	ND
ABJ324	1	32	32	8	64	32	16	16	256	>256	64	-	-	-	0.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ324-1	64	256	512	128	64	128	16	256	256	>256	64	+	0.1	-	2.6	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ329	1	32	32	16	32	>128	8	32	128	>256	64	+	-	19.8	7.8	-	Val44dup
ABJ329-1	32	128	32	32	64	>128	8	32	128	>256	64	+	-	0.5	2.1	-	Val44dup
ABJ331	1	32	128	8	>64	16	8	>512	256	>256	64	-	2.2	10.3	2.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
ABJ331-1	256	256	512	128	> 64	128	8	>512	256	>256	64	+	0.1	2.4	3.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr

^aThe isolates with “-” are the isogenic triclosan-resistant mutant derivatives of the corresponding strains.^bValues in boldface indicate MICs that are increased by at least 4-fold in the triclosan-resistant mutants compared to their parental strains.^c+, -, denote the presence or absence of the relevant genes by RT-PCR, respectively.^d-, no expression.^e-, no mutation found; ND, not detected.

Abbreviation: TCS, triclosan.

Discussion

A. baumannii is considered a significant nosocomial pathogen in veterinary medicine (van der Kolk et al., 2019). This study highlights the common isolation of *A. baumannii* in diseased animals. The proportion of *A. baumannii* clinical isolates from dogs (15.18%) and cats (17.7%) was higher than previous studies (Belmonte et al., 2014), supporting that *A. baumannii* is a frequent cause of opportunistic infections in animals. In this study, up to 70% of the *A. baumannii* isolates from various diseased animals were MDRAB, which is in agreement with a previous study in Germany (Zordan et al., 2011). The samples originated from the carcasses of diseased animals that were expected to receive antibiotic treatment prior to death. This could explain the high resistance rates observed, in addition to the intrinsic resistance property of the pathogen. However, the medical records and history of antibiotic use were not revealed.

All the isolates were resistant to CHL, and high resistance rates to TMP (96.67%) and ERY (93.33%) were observed. This is partly involved in AdeIJK, of which expression was detected in all isolates. The role of AdeIJK in intrinsic resistance to these drugs in *A. baumannii* was previously demonstrated (Rosenfeld et al., 2012). A former study showed that AdeABC expression was limited to *A. baumannii* and commonly found among MDRAB clinical isolates (Chu et al., 2006). This is in agreement with the observation of this study, where most MDRAB (53%) expressed AdeABC in addition to AdeIJK. This observation is also consistent with previous studies (Huys et al., 2005; Nemec et al., 2007) and supports the involvement of AdeABC in the multidrug resistance phenotype of the pathogen. In contrast, the expression of AdeDE was not detected in any isolates in this collection, supporting the notion that expression of this efflux pump is mainly detected in *Acinetobacter* GDG3 (Chu et al., 2006). Taken together, our results demonstrated the wide distribution of Ade multidrug efflux pumps among the *A. baumannii* animal isolates. The observations from this study may not describe the actual impact of the Ade pumps on phenotypic resistance level and other resistance mechanisms (e.g., enzymatic modification, drug target mutation) in *A. baumannii*. Further experiments (e.g., gene inactivation experiment) are required to elucidate their exact contribution.

Some isolates overexpressed Ade multidrug efflux pumps but did not exhibit resistance to antibiotics that were previously shown to be the pumps' substrates. For example, ABJ302 overexpressed AdeG and AdeJ and was not resistant to CIP, TET, and TMP. ABJ328, with a high expression level of AdeG, was susceptible to TET. Therefore, the expression of Ade systems does not always correspond to resistance phenotype, indicating the complexity of resistance mechanisms in the bacterial species. In these isolates, expression of the Ade pumps may contribute to reduced antibiotic susceptibility at a certain level. However, their susceptibility level, as defined by the MIC value, is lower than that of clinical breakpoints. The strains are still defined as susceptible despite the high transcription level of the efflux pumps.

A previous study showed that mutation in AdeL could lead to overexpression of AdeFGH (Coyne et al., 2010), inconsistent with this study, where only one isolate (ABJ302) contained AdeL mutation with 108.4-fold overexpression of AdeG. Several patterns of AdeN regulatory mutation were observed in the isolates with different transcription levels of *adeJ* (2.0 to 16.7-fold). However, among the AdeJ overexpression isolates, ABJ303 overexpressing AdeIJK (6.3-fold) was lacking mutation in AdeN. According to these results, the existence of regulatory mechanisms responsible for the overexpression of AdeFGH and AdeIJK, in addition to mutations in AdeL and AdeN, is suggested. A similar phenomenon was observed in the *P. aeruginosa* clinical isolates, of which the expression of multidrug efflux pumps is not always associated with the mutations in the adjacent regulatory genes (Chuanchuen et al., 2008).

Reserpine and CCCP are non-specific efflux pump inhibitors that have been widely used to screen the phenotypic effect of multidrug efflux pumps in several bacterial species, e.g., *P. aeruginosa*, *Salmonella* (Piddock et al., 2000; Adabi et al., 2015). Reserpine is a competitive inhibitor of both ATP-dependent and proton-dependent transporters (Akiyama et al., 1988), while CCCP is an energy uncoupler that inhibits the function of proton motive force-driven transporters (Bentabollet and Kepes, 1977). In this study, resistance rates of eight and nine antibiotics were decreased as a result of the addition of either reserpine (3-26%) or CCCP (3-26%), respectively, of which the effect of CCCP was more pronounced than reserpine (Table 3). However, the presence of reserpine and CCCP did not affect CIP MIC in any isolates, suggesting that multidrug efflux pumps do not significantly contribute to CIP resistance in MDRAB in this collection. Another mechanism-mediated CIP resistance exists (e.g., mutations in *gyrA* and *pacC*) but was not examined in this study.

The multidrug efflux system has been reported as a major mechanism responsible for cross-resistance between biocides (i.e., BZK, TCS) and antibiotics in *A. baumannii* (Fernando et al., 2014). In this study, triclosan-resistant mutants were obtained after exposure to TCS, while none of the isolates developed resistance after exposure to BZK and CHX. Selection of spontaneous mutants by TCS was previously demonstrated in *P. aeruginosa* and *Salmonella enterica* (Chuanchuen et al., 2001; Gradel et al., 2005). Exposure to TCS resulted in the expression of *abeM* in three isolates (ABJ311-1, ABJ324-1, and ABJ331-1). To date, an association between the *abeM* expression and TCS resistance phenotype in *A. baumannii* has never been reported (Fernando et al., 2014). *E. coli* expressing AbeM showed a significant increase in MICs for norfloxacin, ofloxacin, CIP, and TCS (Su et al., 2005). However, ABJ311-1, a triclosan-resistant derivative expressing AbeM, did not develop resistance to CIP and other antibiotics tested. The role of AbeM in antibiotic resistance is unclear and should be a topic of further study. In addition, the expression of the RND efflux system is not the sole mechanism responsible for acquired resistance to TCS in these *A. baumannii* from animal origins. Other mechanisms (e.g., *fabI* mutation that is well recognized as a responsible mechanism for acquired resistance to TCS in *E. coli* (McMurphy et al.,

1998)) may be involved in TCS resistance in the *A. baumannii* clinical isolates, but their contribution has not been examined in this study yet. Regardless, the results in the present study confirmed that exposure to TCS can promote cross-resistance to antibiotics in *A. baumannii* from animal origins, similar to previous observations in human isolates (Lin *et al.*, 2017).

Unexpectedly, the expression of AdeG and AdeJ in almost all isolates was reduced or turned to be negative after exposure to TCS. This downregulated expression of AdeG and AdeJ may be a stringent response to the stress of triclosan and suggests that these transporters are involved in the stress response mechanisms. A similar phenomenon was demonstrated in a global transcription response to tigecycline in *A. baumannii*, where the downregulated ABC transporters were observed (Hua *et al.*, 2014). Despite the significant reduction of AdeG expression level, ≥ 4 -fold increase in MICs was developed for ATM, CAR, TAZ, CIP, ERY, and PIP in ABJ303-1, ABJ316-1, and ABJ317-1. These findings suggested that the MIC values in these isolates were not solely dependent on AdeFGH expression, and other resistance mechanisms were uncharacterized in this study.

Interestingly, the amino acid substitution Ile145-Val in AdeL of ABJ302 disappeared in its triclosan-resistant mutant ABJ302-1 derivative, but AdeG transcription was reduced from 108.4 fold in ABJ302 to 3.7 fold in ABJ302-1. AdeL mutations were previously shown to be associated with AdeFGH overexpression (Coyne *et al.*, 2010). Reduction of *adeG* expression levels was observed in other triclosan-resistant mutant derivatives (e.g., ABJ311-1, ABJ319-1, ABJ320-1, ABJ321-1, ABJ329-1, ABJ331-1) with no AdeL mutation. In addition, some isogenic parental strain and their triclosan-resistant mutants carried the same mutations in AdeN, but the AdeJ expression was reduced in the derivative mutants (e.g., ABJ311/ABJ311-1, ABJ319/ABJ319-1, ABJ322/ABJ322-1). Once again, these observations suggested that the transcription of *adeG* or *adeJ* is not always a result of mutation in the corresponding regulatory genes. The overexpression of multidrug efflux pumps can be associated with the altered expression of global regulators such as *marA*, *ramA*, or *soxS* that has been recognized in *E. coli* and *Salmonella* Typhimurium (McMurry *et al.*, 1998; Webber *et al.*, 2008). In *A. baumannii*, the H-NS global regulator has been shown to contribute to the multidrug resistance phenotype by regulating the expression of multidrug transporters of the RND family (Geisinger *et al.*, 2018). Recently, a previous study reported BfmRS as a global transcriptional regulator of a post-exponential-phase-like program that is associated with antibiotic resistance, pathogenicity, envelop morphogenesis, and stress response (Deveson Lucas *et al.*, 2018). Since the *A. baumannii* from animal origins responded to exposure to triclosan by altering the expression of multidrug efflux systems, global transcriptional regulators and responses are worth further studies.

In conclusion, the results highlighted that MDRAB is common among diseased animals, indicating the need for routine AMR monitoring in bacteria causing nosocomial infections in animals. The widespread RND efflux systems among the MDRAB from animal

origins were observed, and the involvement of other mechanisms in addition to the RND pump was suggested. Cross-resistance between TCS and antibiotics was predominant but may be independent of the expression of AdeABC, AdeFGH, AdeIJK, AbeM, and AmvA. The regulation of the expression of Ade multidrug systems was complicated. Further studies are required for a better understanding of the expression of RND efflux systems and their regulatory mechanisms and cross-resistance to antibiotics. Prudent use of biocides (i.e., antiseptics and disinfectants) should be encouraged to optimize their efficacy and minimize the development and spread of AMR.

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