

Assessment of Efflux Activity Using H33342 Accumulation in Tigecycline-Resistant *Acinetobacter baumannii* Clinical Isolates

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Background: Tigecycline resistance has emerged recently and has shown diverse mechanisms. The aim of this study was to assess the role of efflux activity in tigecycline resistance in 120 clinical isolates of *A. baumannii* using two methods: the H33342 accumulation assay and *adeB* real-time reverse transcriptase polymerase chain reaction. In addition, we analyzed the correlation between the expression level of *adeB* and H33342 accumulation level.

Methods: *A. baumannii* clinical isolates was divided into tigecycline-resistant (49 strains), intermediate (40 strains), and susceptible (31 strains) groups. The H33342 accumulation was measured in the absence or presence of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Real-time RT-PCR was performed to determine the relative expression of the *adeB* gene in *A. baumannii* clinical isolates.

Results: The level of H33342 accumulation in the resistant group was relatively lower than those in the other groups. The addition of CCCP caused a significantly increased fold change in H33342 accumu-

lation in the tigecycline-resistant group. Significant difference in the fold change level in H33342 accumulation was found between tigecycline-susceptible and resistant isolates. Those findings support the role of efflux pumps of which substrates are H33342 in the resistance of tigecycline. Significant differences in the relative expression levels of *adeB* were shown between tigecycline-susceptible and resistant groups also.

Conclusion: The results showed that several efflux pumps of which substrates were H33342 can contribute to tigecycline resistance. The *adeB* overexpression can also contribute to tigecycline resistance. It is possible that efflux pumps other than *adeB* efflux pumps contribute to tigecycline resistance because there was no correlation between fold change level in H33342 accumulation and *adeB* expression level. (Ann Clin Microbiol 2017;20:90-96)

Key Words: *Acinetobacter baumannii*, Efflux pump inhibitor, Tigecycline

INTRODUCTION

Acinetobacter baumannii has successfully become a significant nosocomial agent worldwide, essentially because of its ability to survive in the hospital environment, and its propensity to rapidly acquire antibiotic resistance mechanisms [1]. In addition to its high capacity to acquire new genetic determinants, overproduction of efflux pumps with broad substrate specificity

has been shown to be associated with multidrug resistance (MDR) in *A. baumannii* [2,3]. The resistance-nodulation-cell division (RND) systems are the most prevalent efflux pump systems in multidrug-resistant *A. baumannii*. Among three RND type efflux pumps, overexpression of AdeABC constitutes a major mechanism of multiresistance in *A. baumannii* [2].

Tigecycline, a glycylcycline antibiotic, retains activity against a broad range of both gram-positive and gram-negative bacteria

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that includes methicillin-resistant *Staphylococcus*, vancomycin-resistant *Enterococcus*, and *A. baumannii* multidrug-resistant isolates [4]. However, tigecycline resistance in *A. baumannii* has emerged recently. It is reported that tigecycline nonsusceptibility in *A. baumannii* isolates has been associated with over-expression of a variety of efflux pumps. Among them, the active efflux pump AdeABC appeared to play important roles in the tigecycline resistance of *A. baumannii* [5].

Rapid assessment of efflux pump activity is important because increased expression of chromosomal genes for efflux systems plays a major role in multidrug resistance (MDR) [2]. Fluorescent dyes, such as ethidium bromide and the bis-benzamide dye Hoechst 33342 (H33342), are substrates of efflux pump systems, and can act as reporters of efflux activity. The H33342 accumulation assay is a cheap, relatively rapid, sensitive and specific test for MDR [6]. It has been used for the assessment of cellular permeability and efflux activity in *Salmonella enterica* and *Escherichia coli* [6,7]. H33342 accumulation has also been used in *A. baumannii* to assess the contribution of efflux activity to antibiotic accumulation [8,9]. Addition of the efflux pump inhibitor, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which dissipates the proton motive force required by several efflux pumps, causes a significant increase in H33342 accumulation, suggesting reduced efflux pump activity due to the inhibition of active efflux [9]. Although it was used effectively to assess efflux activity between several strains of bacteria including *S. enterica*, *E. coli* and *A. baumannii* [6,9], it is hard to find out studies using it as an assessing tool of efflux activity in a lot of clinical strains of *A. baumannii*.

In this study, we assessed the role of the efflux system using H33342 accumulation in tigecycline resistance in 120 clinical isolates of *A. baumannii*, including 49 tigecycline-resistant, 40 intermediate, and 31 susceptible strains. We also investigated the effect of the relative expression of the RND efflux pump *adeB* gene on tigecycline resistance in *A. baumannii* clinical isolates. In addition, we analyzed the correlation between the expression level of *adeB* and H33342 accumulation levels.

MATERIALS AND METHODS

1. Bacterial isolates and growth conditions

A total of 120 *A. baumannii* clinical isolates were collected from January 2012 to December 2015 at Chosun University Hospital for use in this study. Bacterial identification was per-

formed using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France). Thereafter, species identification was confirmed by *gyrB* multiplex PCR and *bla*_{OXA-51-like} PCR [10,11]. Antimicrobial susceptibility for tigecycline was also determined using the VITEK 2 system. The 120 *A. baumannii* clinical isolates were divided into 3 groups, 49 (40.8%) tigecycline-resistant, 40 (33.3%) tigecycline-intermediate, and 31 (25.8%) tigecycline-susceptible strains. These strains were isolated from sputum (72 isolates), open pus (15 isolates), whole blood (13 isolates), catheterized urine (6 isolates), random urine (5 isolates), endotracheal intubator (4 isolates), central venous catheter tip (2 isolates), CSF (1 isolate), bile juice (1 isolate), and bronchial washing (1 isolate). We selected two *A. baumannii* tigecycline-susceptible clinical isolates, 15136S and 15986S, and three *A. baumannii* tigecycline-resistant clinical isolates, 17296R, 18167R, and 18997R, for the H33342 accumulation assay, to determine an incubation time showing unchanged fluorescence accumulation. *A. baumannii* ATCC 19606 were used as reference strains. Bacterial strains were grown at 37°C in Luria-Bertani (LB) broth and agar (Difco Laboratories, Detroit, MI, USA). The study was approved by the Ethics in Human Research Committee of Chosun University Hospital (chosun non2016-003).

2. H33342 accumulation assay

The H33342 accumulation assay was carried out as described by Richmond et al. [9], with the following modifications. Logarithmic phase cells were adjusted to an optical density at a wavelength of 600 nm (OD₆₀₀) of 0.5, and then transferred to wells of a black 96-well plate (Corning, Amsterdam, The Netherlands). Wells were inoculated with 180 μ L of each culture, with or without 50 μ M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and then 2.5 μ M H33342 was added to each well. Four replicates of each strain were analyzed per column. The plate was transferred to a fluorescence microplate reader (SpectraMax Gemini XPS; Molecular Devices, Sunnyvale, CA, USA), and fluorescence was measured at 37°C, by using excitation and emission filters of 355 nm and 460 nm, respectively. Each experiment was repeated twice. Heat-killed *A. baumannii* cells served as a positive control. The heat-killed cells rapidly accumulated H33342, and showed the maximal fluorescence level. The H33342 accumulation ratio was calculated by dividing the amount of H33342 accumulation in the presence of CCCP by the amount of H33342 accumulation in the absence of CCCP. It reflects the fold change in H33342 accumulation in

the presence of CCCP and that in the absence of CCCP.

3. Real-time reverse transcriptase polymerase chain reaction

We performed real-time reverse transcriptase PCR (real-time RT-PCR) to examine the relative expression of the *adeB* gene in *A. baumannii* clinical isolates. We obtained DNase-treated RNA from logarithmic phase cells (OD₆₀₀ of 0.6) using a High Pure RNA isolation kit (Roche, Mannheim, Germany). The cDNA was synthesized with SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA, USA) using random primers according to the manufacturer's instructions. Real-time RT-PCR assays were performed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) with an iTaq Universal SYBR Green Supermix (Bio-Rad) according to the user's guide. For the real-time RT-PCR amplification, the following PCR program was used: 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 30 seconds.

In each real-time RT-PCR run, a negative control (reaction mixture without cDNA) was included. RNA controls without reverse transcription were also included to confirm the absence of contaminating DNA in the samples. The *rpoB* gene was used as a housekeeping gene to normalize the expression of the target *adeB* gene. For real-time RT-PCR of the *adeB* gene, we used *adeB*-Q-for (5'-gatatcttgaatctgaaagtcattg-3') and *adeB*-Q-rev (5'-caaagatggtcgcctgtataac-3') as the forward and reverse primers. For the *rpoB* gene, we used *rpoB*-for-1 (5'-ctcactatggtcgtgttgc-3') and *rpoB*-rev-2 (5'-ccaagaaccgaagtcattcg-3'). The normalized expression of *adeB* was calibrated using the expression of

A. baumannii ATCC 19606, which was assigned a value of 1.0. All experiments were performed in triplicate. We considered a threshold cycle (C_t; the lowest cycle number to produce a detectable fluorescent signal) value of 38 as the cut-off for a negative result in real-time PCR [12].

4. Statistical analysis

Statistical differences between each comparison group were determined using Student's t-test in the H33342 accumulation assay and *adeB* relative expression analysis. SPSS statistics 21 program (IBM Corp., Armonk, NY, USA) was used for the statistical analyses. *P* values of <0.05 were considered statistically significant.

RESULTS

To determine the appropriate time to measure H33342 accumulation, we observed the change in level over the time period of the assay in six representative strains of *A. baumannii*, including ATCC 19606 reference strain, two *A. baumannii* tigecycline-susceptible clinical isolates (15136S and 15986S), and three tigecycline-resistant clinical isolates (17296R, 18167R, and 18997R). The level of H33342 accumulation rapidly increased in the early stage, but was not significantly changed 50 min after the addition of H33342 in six strains, which means it had reached a steady state. Therefore, we selected 50 min as the optimum time to measure H33342 accumulation in the *A. baumannii* clinical isolates. The level of H33342 accumulation of

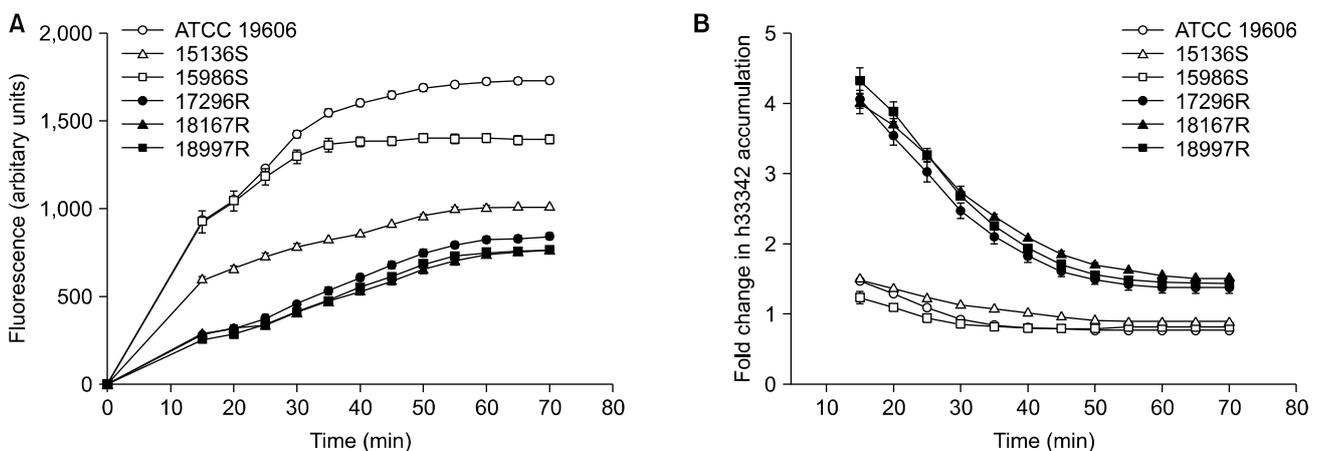


Fig. 1. Pattern of H33342 accumulation (A) and the H33342 accumulation ratio (B) over a 70 min incubation period with 6 *Acinetobacter baumannii* isolates, including ATCC 19606, 2 tigecycline-susceptible (15136S, 15986S), and 3 resistant (17296R, 18167R, 18997R) strains. The H33342 accumulation ratio reflects the fold changes in H33342 fluorescence accumulation after addition of CCCP, relative to basal level without CCCP addition. The error bars represent the standard error of the mean. Abbreviations: H33342, Hoechst 33342; CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

ATCC 19606 and both tigecycline-susceptible strains was higher than that of the three resistant strains at steady-state (Fig. 1A). The addition of CCCP, an efflux pump inhibitor, caused a significant increase in the level of H33342 accumulation only in the tigecycline-resistant strains. The fold change in H33342 accumulation between the presence of CCCP and the absence of CCCP was lower in the tigecycline-susceptible isolates (15136S and 15986S) than in the three tigecycline-resistant isolates (17296R, 18167R, and 18997) (Fig. 1B).

We measured the mean values of H33342 accumulation at steady state (50 min) of the tigecycline-susceptible, intermediate, and resistant groups of 120 clinical isolates. The mean fluorescence of tigecycline-susceptible, intermediate groups and resistant group were 717.0 ± 42.7 , 694.0 ± 32.4 , and 510.3 ± 33.8 , respectively. Addition of CCCP caused a significant increase of fluorescence in the tigecycline-resistant *A. baumannii* group ($P < 0.001$), but not in the susceptible and intermediate groups. The mean fluorescence of the tigecycline-resistant *A. baumannii* group increased from 510.3 ± 33.8 to 659.7 ± 25.9 in the presence of CCCP (Fig. 2A). The mean fold change in H33342 accumulation was 1.46 ± 0.076 for the tigecycline-resistant group, and 0.96 ± 0.04 and 1.02 ± 0.054 for the susceptible and intermediate groups, respectively. Significant difference in fold change level of H33342 accumulation was found between tigecycline-susceptible and tigecycline-resistant group ($P < 0.0001$) (Fig. 2B).

We measured the mean values of the relative expression of *adeB* by real-time RT-PCR in tigecycline-susceptible, intermediate, and resistant groups. All 120 clinical isolates of *A. baumannii* showed positive results for real-time RT-PCR of

adeB gene (Ct value range: 13.23 to 28.18). The mean relative expression values of *adeB* of tigecycline-susceptible, intermediate, and resistant groups were 10.60 ± 1.33 , 14.19 ± 1.19 , and 15.39 ± 1.33 , respectively. The median relative expression values of *adeB* of tigecycline-susceptible, intermediate, and resistant groups were 9.24, 18.35, and 22.0, respectively. We found significant difference in *adeB* expression level between tigecycline-susceptible and tigecycline-resistant group ($P < 0.05$) (Fig. 3).

When we analyzed the correlation between the expression level of the RND efflux pump *adeB* gene and H33342 accumulation levels in 120 *A. baumannii* clinical isolates, we could not

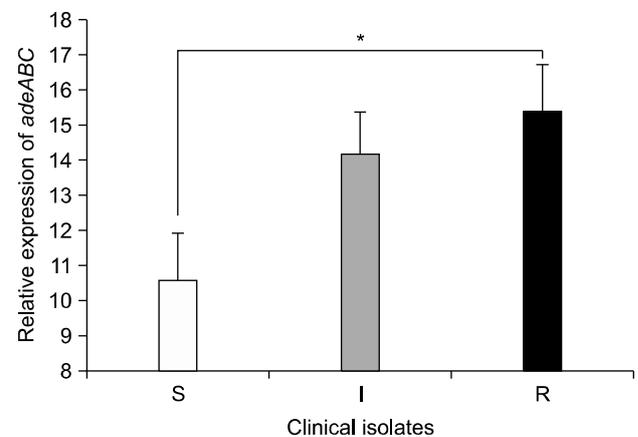


Fig. 3. Relative expression of the efflux pump gene, *adeB*, determined by real-time reverse transcriptase PCR in 31 tigecycline-susceptible (S), 40 intermediate (I), and 49 resistant (R) *Acinetobacter baumannii* strains. The normalized expression of *adeB* was calibrated with the expression of *A. baumannii* ATCC 19606. The error bars represent the standard error of the mean. * $P < 0.05$.

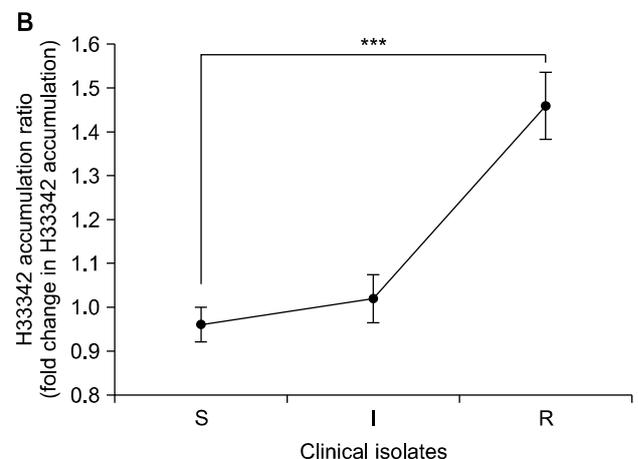
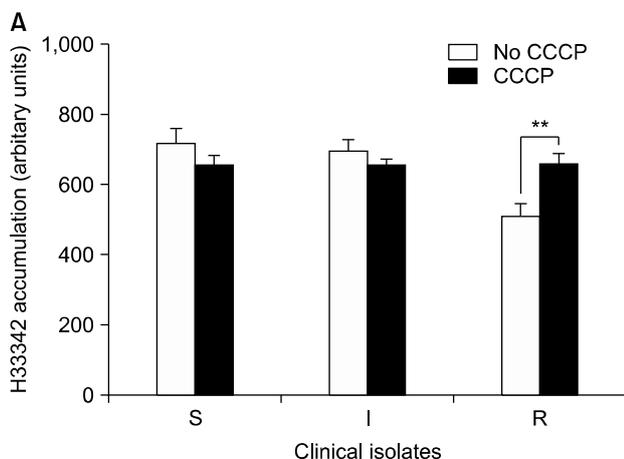


Fig. 2. Comparison of the mean values of H33342 accumulation in the presence and absence of CCCP (A) and the H33342 accumulation ratio (B) of tigecycline-susceptible (S), intermediate (I), and resistant (R) groups in 120 *Acinetobacter baumannii* clinical isolates at 50 min of exposure (steady state). The error bars represent the standard error of the mean. ** $P < 0.001$; *** $P < 0.0001$. Abbreviations: H33342, Hoechst 33342; CCCP, carbonyl cyanide 3-chlorophenylhydrazone.

find any correlation between fold change level in H33342 accumulation and *adeB* expression level.

DISCUSSION

Treatment of *A. baumannii* has become a major concern in hospital settings due to the wide dissemination of multi-drug-resistant strains. Overexpression of efflux pumps in *A. baumannii* is a common mechanism of multidrug resistance in this nosocomial pathogen [2].

The H33342 accumulation assay was introduced to estimate the contribution of active efflux pumps to antimicrobial resistance in *Salmonella* Typhimurium strains [6]. The H33342 accumulation assay was also used to assess the roles of efflux pumps in antimicrobial resistance in *A. baumannii* clinical isolates [9].

We utilized the H33342 accumulation assay to assess efflux activity in 120 *A. baumannii* clinical isolates. In this study, the tigecycline-susceptible and intermediate groups showed relatively higher H33342 accumulation than the tigecycline-resistant group at steady-state. The addition of efflux pump inhibitor CCCP caused a statistically significant increase in H33342 accumulation only in the tigecycline-resistant group. These findings suggest the role of efflux activity in tigecycline resistance based on the following explanations: 1) the strains belonging to the tigecycline-resistant group showed low H33342 accumulation due to effective expulsion of H33342 by active efflux pumps; and 2) increased H33342 accumulation with the addition of CCCP only in the tigecycline-resistant group supports the role of efflux in tigecycline-resistance in that group.

The addition of efflux pump inhibitors (EIs) highlighted the contribution of various efflux pumps to antimicrobial resistance in a variety of bacteria [6,9,13]. The addition of PA β N, which inhibits RND transporters, resulted in increased H33342 accumulation, i.e. reduced levels of efflux [9]. Whalen et al. [13] reported that 3,4-dibromopyrrole-2,5-dione, an inhibitor of RND transporters, decreased the minimum inhibitory concentrations (MICs) of various antibiotics, and also increased the intracellular accumulation of H33342 in wild-type, but not in transporter-deficient strains. Opperman et al. [7] reported MBX2319, a novel pyranopyridine inhibitor, had potent activity against RND efflux pumps, and also increased intracellular accumulation of H33342 in wild-type, but not AcrAB-TolC-deficient strains. As described above, the addition of CCCP in *A. baumannii* AYE reference strain caused a significant increase in H33342 accumulation, suggesting reduced efflux [9]. In that

study, the *adeB* mutant showed increased H33342 accumulation, indicative of reduced activity compared to the parental strain. Moreover, the addition of CCCP caused a significant increase ($P < 0.05$) in the level of H33342 accumulation for all 6 clinical isolates tested [9].

The AdeABC pump is present in approximately 80% (53% to 97%) of *A. baumannii* clinical strains [2,14]. Our study showed the presence of the *adeB* gene in all 120 *A. baumannii* clinical isolates. Deng et al. [5] reported that the positivity rate of *adeB* was 84.4% (54/64) for tigecycline-nonsusceptible *A. baumannii* (TNAB) isolates, and 80% (8/10) for tigecycline-susceptible *A. baumannii* (TSAB) isolates. These results were contrary to an earlier result showing that *adeABC* genes were present in MDR isolates, and not in susceptible isolates [15].

It is reported that reduced susceptibility to tigecycline appears to be mediated in part by active efflux systems, including AdeABC. In this study, the mean relative expression of the *adeB* gene of tigecycline-susceptible, intermediate, and resistant groups was 10.60 ± 1.33 , 14.19 ± 1.19 , and 15.39 ± 1.33 , respectively. We found significant differences in the levels of *adeB* relative expression between tigecycline-susceptible versus tigecycline-resistant groups ($P < 0.05$). Therefore, it seems possible that *adeB* overexpression may contribute to tigecycline resistance.

Deng et al. [5] reported that the mean expression level of the *adeB* gene in 64 TNAB clinical isolates was 29-fold higher than that in 10 TSAB isolates. Peleg et al. [16] identified 40-fold and 54-fold increases in *adeB* expression in the two TNAB isolates compared to that in a TSAB isolate. Although we do not know the exact cause of the large differences in *adeB* expression level between our strains and Deng's, several factors may contribute the differences between them. In contrast to Deng's strains, collected for 1 year, our strains, randomly collected for almost 4 years, may have relatively diverse results, which may explain the difference between them. In addition, we suspect the different features of the test strains, including resistance determinants harbored in each strain, biological state, strain distribution in tested bacteria, characteristics of prevailing or endemic clones in each region or country, the housekeeping gene or control strain used for calibration of *adeB* relative expression, and the measurement method, condition or equipment etc., may explain the differences in *adeB* relative expression. Such a difference was also observed in the reported incidence of *adeB* overexpression. Yoon et al. [17] reported significant *adeB* overexpression in 76.9% (10/13) of TNAB clinical strains containing the *adeB* gene. Correlation between *adeB* overexpression and reduced

susceptibility to tigecycline was observed in 5/40 (12.5%) MDR *A. baumannii* clinical isolates [18]. Rumbo et al. [14] reported that reduced susceptibility to tigecycline was associated with overexpression of different efflux pump genes, such as AdeABC or AdeIJK, or the presence of TetA efflux pumps, according to the tested clone.

No correlation between the expression level of the RND efflux pump *adeB* gene and fold change of H33342 accumulation was found in 120 *A. baumannii* clinical isolates. Although we don't know the reason for that, it may be related to the difference in the number of involved efflux pumps resulting in each determinant. Any efflux pumps, of which the substrates include H33342, may exert influence on the H33342 accumulation levels. Because H33342 accumulation levels may be determined by the combined activity of a variety of efflux pumps including *adeB*, it could not be correlated with the single *adeB* gene expression level.

The limitation of the present study is that the overall perspective for contribution factor to tigecycline resistance could not be obtained because this study is focused on H33342 accumulation assay and the relative expression of the *adeB* gene. Therefore, it may be necessary to undertake further study to closely evaluate their correlation by measuring other relevant factors together. The study may encompass another RND system or a variety of efflux pump genes which may contribute the tigecycline resistance.

In conclusion, our study showed that efflux activity, assessed by H33342 accumulation assay, may contribute to tigecycline-resistance in *A. baumannii* clinical isolates. However, there was no correlation between the H33342 accumulation ratio and *adeB* expression level.

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=국문초록=

Tigecycline 내성 *Acinetobacter baumannii* 임상 분리주들에서 H33342 축적에 의해 측정된 유출펌프 활성의 평가

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배경: Tigecycline 내성이 최근 부상하고 있고 tigecycline 내성과 관련된 기전은 다양하다. 본 연구에서는 *A. baumannii*의 임상 분리주 120주에서 H33342 축적 분석법과 *adeB* 유전자의 실시간 역전사 효소 중합 효소 연쇄 반응법의 두 방법을 사용하여 tigecycline 내성에 있어서 유출펌프 활성의 역할을 평가하고자 하였다. 또한 *adeB* 유전자의 발현 수준과 H33342 축적 수준 사이의 상관 관계를 분석 하였다.

방법: *A. baumannii* 임상 분리주들은 tigecycline 내성(49주)과 중간내성(40주), 및 감수성(31주)군으로 분류하였다. H33342 축적수준은 유출 펌프 억제제인 carbonyl cyanide 3-chlorophenylhydrazone (CCCP)를 첨가하기 전과 후에 각각 측정하였다. *A. baumannii* 임상 분리주들에서 *adeB* 유전자의 상대적인 발현을 결정하기 위해 실시간 역전사 효소 중합 효소 연쇄 반응법이 수행되었다.

결과: Tigecycline 내성군에서의 H33342 축적 수준은 다른 군들의 축적수준보다 상대적으로 더 낮았다. Tigecycline 내성군에서 H33342 축적 수준은 CCCP를 첨가 한 후 크게 증가된 변화값(fold change)을 보였다. Tigecycline 감수성군과 내성군간에 H33342 축적수준의 변화값은 유의한 차이를 보여주었다. 이러한 소견들은 유출 펌프의 기질들 중에 H33342가 포함되어 있는 유출펌프들이 tigecycline 내성에 역할을 한다는 것을 뒷받침해준다. 또한 tigecycline 감수성군과 내성군간에 *adeB* 유전자의 상대적인 발현 수준에 유의한 차이가 나타났다.

결론: 이들 연구결과들은 H33342가 기질인 여러 유출 펌프가 tigecycline 내성에 기여할 수 있음을 보여 주었다. 또한 *adeB* 유전자의 과발현이 tigecycline 내성에 기여할 수 있다는 것을 보여준다. H33342 축적의 변화값 수준과 *adeB* 유전자 발현 수준간에는 상관 관계가 없었기 때문에 *adeB* 유출 펌프 이외의 유출 펌프가 tigecycline 내성에 기여할 수 있다고 보는 것이 가능하다. [Ann Clin Microbiol 2017;20:90-96]

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