**Brief communication**

# Screening antibiotics using an Hoechst 33342 dye-accumulation assay to detect efflux activity in *Acinetobacter baumannii* clinical isolates

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## Abstract

**Background:** Understanding the contribution of efflux pumps to the resistance of antibiotics is useful when considering strategies for antimicrobial therapy.

**Objectives:** To assess the role of efflux activity on the resistance of antibiotics commonly used in hospitals.

**Methods:** We analyzed the efflux activity of 120 clinical isolates of *Acinetobacter baumannii* using an Hoechst 33342 (H33342) dye-accumulation assay. We compared the indicators for efflux activity of susceptible and non-susceptible groups of each of 16 tested antibiotics. To determine the role of efflux activity on resistance to an antibiotic, we used 3 criteria based on the results of the H33342-accumulation assay.

**Results:** The evaluation suggests that efflux activity contributed to resistance to the following 11 antibiotics: cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, and tigecycline. However, ampicillin/sulbactam, minocycline, and trimethoprim/sulfamethoxazole did not meet the criteria, suggesting resistance may not be mediated by efflux activity. A significant difference in efflux activity was observed between bacteria belonging to the multidrug-resistant *Acinetobacter baumannii* (MDRAB) group and those belonging to the non-MDRAB group.

**Conclusions:** Efflux activity may contribute to multidrug resistance and particularly resistance to numerous antibiotics used in hospitals. These antibiotics would be good candidates for combination therapeutic regimens consisting of an antibiotic and an efflux pump inhibitor as an adjuvant to combat drug efflux.

**Keywords:** antibacterial agents, bacterial, drug resistance, efflux, Hoechst 33342, multiple, role

*Acinetobacter baumannii* is a nosocomial pathogen of increasing importance because of its resistance to multiple antibiotics [1]. Efflux pumps of the resistance-nodulation-division superfamily play a major role in multidrug resistance by actively excreting a very wide range of antimicrobial agents. These efflux pumps are of considerable interest not only because of their role in drug resistance but also because they are targets for novel adjunct therapies [2]. Recently, research to characterize resistance-modifying efflux pump inhibitors (EPIs) that block drug extrusion, thereby restoring antibacterial susceptibility, has increased [2].

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Using drug combinations is one of the methods to effectively control the multidrug-resistant (MDR) organisms [3]. Such combinations include antibiotic–antibiotic combinations and the pairing of an antibiotic with a nonantibiotic adjuvant molecule such as an EPI to directly target resistance mechanisms [3]. EPIs such as phenylalanine-arginine β-naphthylamide (PaβN) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) noticeably decreased the MICs of various antibiotics [4, 5]. Thus, they restored susceptibility to antibiotics that are well-known substrates of efflux pumps [4]. Therefore, EPIs can improve the efficacy of antibiotics and ameliorate the crisis in health care caused by the multidrug resistance of Gram-negative pathogens [6]. In the future, EPIs might be included in therapeutic regimens for *Acinetobacter* to suppress efflux activity for better efficacy. If efflux activity contributes to resistance against some antibiotics, those antibiotics could be candidates for such therapeutic regimens [6]. Thus, it would be helpful to know which antibiotics are affected by efflux activity to select candidate drugs for combination therapies to suppress efflux activity.

If we want to know whether an efflux pump mechanism contributes to the resistance of the specific antibiotic to the bacteria, monitoring the level of accumulation of the antibiotics after the addition of EPI may provide the clue for that. Previously, the cellular accumulation of antibiotics has been assessed directly using radiochemical and fluorescent techniques that monitor accumulation of specific antibiotics [7]. However, these procedures can be time consuming because separation of bacterial cells and the medium is required [7]. To solve this problem, fluorescent compounds, such as Hoechst 33342 (H33342) and ethidium bromide, were used as surrogate markers to assess efflux activity instead of antibiotics [7, 8]. These fluorescent intercalators change their wavelength of maximal emission in different environments when intercalating with DNA, and this phenomenon has been exploited to enable discrimination between intra- and extracellular localization of the probe [7].

In conjunction with EPIs, the H33342 accumulation assay can be used to assess the contribution of efflux pumps to an MDR phenotype [8] and to the resistance to several antibiotics. H33342, a bis-benzamide fluorescent dye, is readily taken up by living cells and fluoresces upon binding to DNA in the hydrophobic environment of the lipid membrane [8]. If antibiotics and H33342 are the substrates of the same efflux pump system, their pattern of accumulation may be similar. In these conditions, the changing pattern of H33342 accumulation after the addition of EPI may be duplicated by these antibiotics. Therefore, H33342 is adopted as a reporter of accumulation and efflux activity [7].

Because H33342 is a substrate of several efflux pumps, it can be pumped out of bacteria by these pumps. The amount of H33342 accumulation is lower in bacteria having high efflux activity than in bacteria having low efflux activity [8]. The addition of EPI CCCP, which dissipates the proton motive force required by several efflux pumps, may cause a significant increase in H33342 accumulation in bacteria with active efflux [8].

Although the H33342 accumulation assay is useful in assessing efflux activity, it is difficult to find studies that determine which antibiotics are affected by efflux pumps using this method. The aim of the present study is to assess the role of efflux activity on antimicrobials commonly used in hospitals by applying the H33342 accumulation assay to clinical isolates of *A. baumannii*.

## Materials and methods

### Bacterial isolates

After approval by the institutional review board (IRB) of Chosun University Hospital (approval No. NON2016-003), 120 anonymized clinical isolates of *A. baumannii* were selected unsystematically from those collected at Chosun University Hospital from January 2012 to December 2015 as part of routine care. The IRB specifically waived any need for informed consent because the isolates were anonymized to the investigators and the study did not involve investigators interacting or intervening with living individuals for research purposes to obtain the isolates as compliant with the U.S. Department of Health and Human Services' regulations for the protection of human subjects (section 45 CFR 46.102(f)). *A. baumannii* was initially identified using the VITEK 2 system (bioMérieux). The identification of species was verified using $bla_{OXA51}$-like polymerase chain reaction (PCR) tests [9] and gyrB multiplex PCR [10]. Antimicrobial susceptibility to the 16 antibiotics, as determined by the VITEK 2 system, was used to allocate isolates into groups. The following antibiotics were tested: aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins plus β-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins plus β-lactamase inhibitors, polymyxins, and tetracyclines.

The 120 *A. baumannii* strains were divided into 2 groups (susceptible and nonsusceptible) based on antimicrobial susceptibility results for each antibiotic. The strains showing intermediate or resistant phenotypes against each antibiotic were included in the nonsusceptible group. Additionally, the strains were divided into multidrug-resistant *A. baumannii* (MDRAB) (100 isolates) and non-MDRAB groups (20 isolates) according to the determination of the antimicrobial susceptibility patterns.

MDRAB was defined as a strain that was nonsusceptible to at least 1 agent in each of 3 or more antimicrobial categories described previously [11]. Non-MDRAB was defined as a strain that was susceptible to all antibiotics or non-susceptible to antibiotics in fewer than 3 categories. Strain ATCC19606$^T$ was used as a reference strain. Bacteria were grown at 37°C in Luria–Bertani (LB) broth and agar (Difco Laboratories). All chemicals used were purchased from Sigma–Aldrich Co.

### H33342 accumulation assay

We performed the H33342 accumulation assay to assess efflux activity of each strain. We compared the H33342 accumulation ratios (HARs) in the following groups: (i) susceptible vs. nonsusceptible for each antibiotic and (ii) MDRAB vs. non-MDRAB.

The H33342 accumulation assay was conducted as described by Richmond et al. with the following modifications [8]. Logarithmic phase cells were adjusted to an OD$_{600}$ of 0.5 and then transferred to wells of black 96-well plates (Corning). The wells of the black microtiter plates were inoculated with 180 μL of each culture with or without 50 μM CCCP, and then 2.5 μM H33342 was added to each well. Four replicates for each strain were analyzed. Fluorescence was read on a fluorescence microplate reader (SpectraMax Gemini XPS; Molecular Devices) at 37°C 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 maximum fluorescence.

The HAR was calculated by dividing the amount of H33342 accumulation in the presence of CCCP (HAC) by the amount of H33342 accumulation in the absence of CCCP (HA).

Efflux activity was considered a contributor to antibiotic resistance if all 3 of the following criteria were met: (1) a significant difference in the mean HAR was noted between the susceptible group and nonsusceptible group for an antibiotic; (2) the mean HA in the nonsusceptible group was lower than that in the susceptible group for an antibiotic; and (3) in the nonsusceptible group, the mean HA was lower than the mean HAC for an antibiotic.

## Results

The results of the H33342 accumulation assay as an indicator of efflux activity are shown according to susceptibility to each antibiotic (**Table 1**).

We compared these results to determine which antibiotics met the criteria, suggesting a role for efflux activity in resistance to the antibiotic. The following 11 of 16 antibiotics met all 3 criteria, suggesting that efflux activity contributed to resistance to these antibiotics: cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, and tigecycline. The numbers of antibiotics that met the first, second, and third criteria were 11 (69%), 13 (81%), and 15 (94%) of the 16 antibiotics tested, respectively. The mean HAC was subtracted from the mean HA in the susceptible and nonsusceptible groups of each antibiotic. The difference between them was a negative quantity in 15 of 16 antibiotics tested in the nonsusceptible group, because the mean HA was lower than the mean HAC in these cases. The difference between these values was positive in 12 of 16 antibiotics tested in the susceptible group, because the mean HA was higher than the mean HAC in these cases (**Table 1**).

The following 5 of 16 antibiotics did not meet criteria 1, 2, or 3: ampicillin/sulbactam, aztreonam, colistin, minocycline, and trimethoprim/sulfamethoxazole (**Table 1**). The mean HA in the nonsusceptible group was higher than that in the susceptible group for aztreonam, trimethoprim/sulfamethoxazole, and colistin. In both the susceptible and nonsusceptible groups, the mean HA was lower than the mean HAC for ampicillin/sulbactam, minocycline, and trimethoprim/sulfamethoxazole. This suggests that the difference between susceptible and nonsusceptible groups is not significant. We could not assess the role of efflux activity on resistance to aztreonam and colistin because the number of strains allocated to the susceptible and nonsusceptible groups was highly disproportionate, with only one strain susceptible to aztreonam and one strain nonsusceptible to colistin included in each group.

## Discussion

The present study on the role of efflux activity in resistance to each antibiotic assessed by the H33342 accumulation assay suggests that efflux activity in clinical isolates of *A. baumannii* may contribute to resistance against cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, and tigecycline. Most of the antibiotics described earlier are known to be the substrates of efflux pumps such as AdeABC and AdeIJK. β-Lactam drugs, including aztreonam, ticarcillin, and minocycline, and all cephalosporins are substrates for AdeIJK [1, 12]. Ampicillin, ceftazidime, gentamicin, and imipenem are substrates for AdeABC [1]. Ceftazidime, cefepime, meropenem, and tigecycline are substrates for

**Table 1.** Results of Hoechst 33342 dye-accumulation assays of 120 *Acinetobacter baumannii* clinical isolates according to the susceptibility and nonsusceptibility of 16 antibiotics

| Antibiotic(s) | Group | No. | HA | | | HAC | | | D (HA − HAC)[†] | HAR[‡] | | | Suitability to each criterion[§] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | P | Mean | SD | P | | Mean | SD | P | C1 | C2 | C3 | AC |
| Cefepime | Susceptible | 19 | 742.1 | 223.1 | 0.028 | 685.1 | 164.9 | 0.447 | 57.03 | 0.9 | 0.2 | 0.000 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 101 | 607.5 | 245.4 | | 655.3 | 154.4 | | −47.81 | 1.2 | 0.5 | | | | | |
| Cefotaxime | Susceptible | 17 | 740.8 | 242.0 | 0.042 | 696.9 | 172.5 | 0.295 | 43.94 | 1.0 | 0.3 | 0.010 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 103 | 610.4 | 243.0 | | 654.0 | 152.8 | | −43.61 | 1.2 | 0.5 | | | | | |
| Ceftazidime | Susceptible | 19 | 742.1 | 223.1 | 0.028 | 685.1 | 164.9 | 0.447 | 57.03 | 0.9 | 0.2 | 0.000 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 101 | 607.5 | 245.4 | | 655.3 | 154.4 | | −47.81 | 1.2 | 0.5 | | | | | |
| Imipenem | Susceptible | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |
| Meropenem | Susceptible | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |
| Piperacillin | Susceptible | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |
| Piperacillin/ tazobactam | Susceptible | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |
| Ticarcillin/ clavulanic acid | Susceptible | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |
| Ampicillin/ sulbactam | Susceptible | 25 | 675.4 | 237.5 | 0.289 | 694.4 | 169.0 | 0.216 | −19.00 | 1.1 | 0.4 | 0.387 | 0 | 1 | 1 | 0 |
| | Non-susceptible | 95 | 616.6 | 248.1 | | 651.0 | 151.7 | | −34.42 | 1.2 | 0.5 | | | | | |
| Aztreonam | Susceptible | 1 | 487.5 | | NA | 485.1 | | NA | 2.34 | 1.0 | | NA | 0 | 0 | 1 | 0 |
| | Non-susceptible | 119 | 630.0 | 246.9 | | 661.5 | 155.5 | | −31.49 | 1.2 | 0.5 | | | | | |
| Tigecycline | Susceptible | 31 | 732.0 | 236.9 | 0.006 | 666.1 | 164.7 | 0.804 | 65.92 | 1.0 | 0.2 | 0.000 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 89 | 592.9 | 240.3 | | 657.9 | 153.4 | | −65.04 | 1.3 | 0.5 | | | | | |
| Minocycline | Susceptible | 111 | 636.7 | 250.5 | 0.219 | 656.3 | 147.7 | 0.555 | −19.56 | 1.2 | 0.5 | 0.200 | 0 | 1 | 1 | 0 |
| | Non-susceptible | 9 | 531.6 | 164.6 | | 706.5 | 241.5 | | −174.84 | 1.4 | 0.4 | | | | | |
| Ciprofloxacin | Susceptible | 19 | 742.1 | 223.1 | 0.028 | 685.1 | 164.9 | 0.447 | 57.03 | 0.9 | 0.2 | 0.000 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 101 | 607.5 | 245.4 | | 655.3 | 154.4 | | −47.81 | 1.2 | 0.5 | | | | | |
| Gentamicin | Susceptible | 25 | 700.5 | 218.8 | 0.102 | 682.8 | 156.7 | 0.414 | 17.70 | 1.0 | 0.2 | 0.002 | 1 | 1 | 1 | 1 |
| | Non-susceptible | 95 | 610.0 | 250.5 | | 654.0 | 155.7 | | −44.08 | 1.2 | 0.5 | | | | | |
| Colistin | Susceptible | 119 | 626.6 | 246.0 | NA | 660.9 | 156.1 | NA | −34.25 | 1.2 | 0.5 | NA | 0 | 0 | 0 | 0 |
| | Non-susceptible | 1 | 893.5 | | | 563.1 | | | 330.33 | 0.6 | | | | | | |

(*Continued*)

**Table 1.** Results of Hoechst 33342 dye-accumulation assays of 120 *Acinetobacter baumannii clinical* isolates according to the susceptibility and nonsusceptibility of 16 antibiotics (*Continued*)

| Antibiotic(s) | Group | No. | HA | | | HAC | | | $D$ (HA – HAC)[†] | HAR[‡] | | | Suitability to each criterion[§] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | SD | P | Mean | SD | P | | Mean | SD | P | C1 | C2 | C3 | AC |
| Trimethoprim/ sulfamethoxazole | Susceptible | 31 | 628.2 | 238.9 | 0.979 | 684.4 | 165.3 | 0.276 | −56.23 | 1.2 | 0.4 | 0.767 | 0 | 0 | 1 | 0 |
| | Non-susceptible | 88 | 629.6 | 251.3 | | 649.0 | 151.2 | | −19.39 | 1.2 | 0.5 | | | | | |
| Multidrug resistance | Non-MDR | 20 | 726.4 | 228.3 | 0.052 | 690.4 | 162.2 | 0.342 | 36.00 | 1.0 | 0.3 | 0.004 | 1 | 1 | 1 | 1 |
| | MDR | 100 | 609.3 | 246.0 | | 654.0 | 154.5 | | −44.65 | 1.2 | 0.5 | | | | | |

[†]Calculated by subtraction of the mean HAC from the mean HA: $D$ (HA – HAC) = HA – HAC

[‡]Calculated by dividing the amount of HAC by the amount of HA: HAR = HAC/HA

[§]All 3 criteria (C1–C3) suggest the contribution of efflux activity to antibiotic resistance. Criterion 1 is a significant difference in the mean HAR between the susceptible group and non-susceptible group for an antibiotic. Criterion 2 is a mean HA in the non-susceptible group that is lower than that in the susceptible group for an antibiotic. Criterion 3 is a mean HA that is lower than the mean HAC in the non-susceptible group for an antibiotic. 1 means the criterion is met. 0 means the criterion is not met

AC, all 3 criteria (C1–C3); C1, criterion 1; C2, criterion 2; C3, criterion 3; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; $D$ (HA – HAC), the difference between HA and HAC; H33342, Hoechst 33342; HA, H33342 accumulation in the absence of CCCP; HAC, H33342 accumulation in the presence of CCCP; HAR, H33342 accumulation ratio; SD, standard deviation

AdeABC and AdeIJK [1]. Ciprofloxacin, trimethoprim, and sulfonamide are substrates for AdeABC, AdeIJK, and adeFGH efflux pumps [1, 12, 13]. Ciprofloxacin is a substrate for AbeS [14]. Because the results of the H33342 accumulation assay suggest that efflux activity may contribute to resistance against these antibiotics and they are all known substrates of efflux pumps, efflux activity appears to be strongly associated with resistance in these cases.

By contrast, ampicillin/sulbactam, minocycline, and trimethoprim/sulfamethoxazole did not meet criterion 1 or 2 of the 3 criteria. Therefore, efflux activity did not appear to exert a major effect on resistance to these antibiotics. However, there is the possibility that efflux pump activity contributed to resistance to a minor degree in strains having other stronger resistance mechanisms. Although minocycline is a substrate of adeIJK [1] and trimethoprim and sulfonamide are substrates of adeABC, adeIJK, and adeFGH [1, 12, 13], they did not show significant differences in mean HARs of the susceptible and nonsusceptible groups. The reason for this is not clear. We suspect that other resistance determinants in some test strains might have distorted or masked the usual pattern attributed to efflux pumps with ampicillin/sulbactam, minocycline, or trimethoprim/sulfamethoxazole.

In addition, previous reports have shown various discrepancies in the activity of resistance-modifying EPIs. Thus, the level of antibiotic activity restored by the effect of EPIs will depend on the antibiotic class and type of EPI used [4]. The activity of a specific EPI is reported to differ by antibiotic.

Thus, PaβN, which drastically decreases the MIC of levofloxacin in MexAB-OprM-overproducing *Pseudomonas aeruginosa*, showed very little effect on the MIC of carbenicillin. This could be related to the respective affinity of the ligands (i.e., the EPI or antibiotic molecule) for the pump or with the level of expression of the acting pump under the tested conditions. Moreover, some EPIs can be more effective for a specific efflux pump [4]. Variability in the results of assays for efflux pump activity such as the H33342 or ethidium bromide accumulation assays and that of efficiency of EPIs such as CCCP or PaβN has been observed among bacterial strains [4, 8]. When Richmond et al. [8] compared the results of H33342 accumulation assay with those of ethidium bromide and norfloxacin in assessing efflux activity in clinical isolates of *A. baumannii*, the pattern of accumulation of norfloxacin broadly reflected the accumulation of H33342. However, ethidium bromide showed a different pattern of accumulation. Because the pattern of efflux activity could be expressed differently according to the substrates of efflux pump or EPI used for evaluation, assessing efflux pump activity using more than one substrate of efflux pumps and EPI may be a better assessment.

Not all the efflux pump activity in bacterial strains could be assessed by H33342 accumulation assay because H33342 is not the substrate of the entire pump. We cannot preclude the possibility that H33342 may not be appropriate for screening efflux activity of antibiotics such as minocycline and trimethoprim/sulfamethoxazole. Even if such a situation arises, it may not affect our research goal. Our research goal was to

screen candidate antibiotics useful for combination therapy using EPI. It may be a better strategy to select drugs with a strong efflux activity to ensure a higher effect with combination therapy with EPI. Therefore, selecting the candidate antibiotics among drugs showing high efflux activity may be a good starting point to investigate better combination model.

Although an assessment of the effect of efflux activity on resistance to colistin was not possible in this study because of the disproportionate number of strains allocated to the susceptible and nonsusceptible groups, efflux activity may not contribute to colistin resistance. Colistin, which acts on the surface of cells, has not been identified as a substrate for any efflux pumps [1]. Kuo et al. showed that the MIC value for ampicillin/sulbactam was not changed by the addition of EPIs, which suggested a negligible effect of efflux activity on resistance to this antibiotic [15]. This finding is compatible with our results, suggesting that efflux pumps may not exert a major effect on resistance to ampicillin/sulbactam.

The present study shows significant differences in efflux activity between bacteria belonging to the MDRAB and non-MDRAB groups, which is consistent with previous findings that increased expression of chromosomal genes for efflux systems plays a major role in MDR [1, 12].

The results of the H33342 accumulation assay as a tool to assess efflux activity were presented as the HAR, HA, and HAC in this study (**Table 1**). The number of antibiotics showing significant differences in the HAR, HA, and HAC between susceptible and nonsusceptible groups was 12, 5, and 0, respectively. Although the HARs were significantly different in the MDRAB and non-MDRAB groups, the HA showed borderline statistical differences and the HAC showed no significant difference between the groups. This might indicate that efflux activity may be better represented by the HAR than the HA or HAC. Because the HAR was calculated by dividing the HAC by the HA, it reflects the whole process of H33342 accumulation assessment. Therefore, we adopted the HAR as a criterion to assess the role of efflux activity on resistance to antibiotics. The H33342 accumulation assay can be used as a convenient screening method to select antibiotics that could be considered targets for new therapeutic regimens such as antibiotic–EPI adjuvant combinations. In addition, it can be used to select strains that demonstrate high efflux activity among numerous test bacteria. If strains have only low efflux activity or they harbor several resistance determinants simultaneously, it is difficult to assess the role of efflux pumps because of the compound effects. After several strains having high efflux activity are selected using such a screening method, they can be used as test strains to evaluate efflux activity assessment methods or to determine the role of the efflux pump activity on antibiotic resistance.

In the future investigation, effects of EPIs other than CCCP, preferably specific EPIs against tested pumps, on the efflux pump activity need to be studied to get more detailed information about them. In addition, further in-depth study is needed to elucidate the interrelationships between the following related parameters: (1) structural and physiological features of efflux pumps and interacting ligands such as antibiotics and EPIs to develop better ligands and (2) interactions between efflux pumps and other determinants of resistance to a given substrate (an antibiotic) in MDRAB clinical isolates.

## Conclusion

Efflux activity may contribute to resistance to numerous antibiotics used in hospitals and multidrug resistance. Antibiotics against which resistance is mediated by efflux activity would be good targets for combination therapies to combat drug efflux.

**Conflict of interest statement.** The authors have completed and submitted the ICMJE Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

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