In vitro activity of methylene blue and EMB agar on colistin-resistant *A.baumannii*: An experimental study

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Abstract

Background Colistin is one of the last resort antibiotics used against carbapenem-resistant *Acinetobacter baumannii* (AB); however, colistin resistance has been reported recently. Methylene blue (MB) is used in microbiology for staining, and in medicine as an antidote drug. Here, we investigated antimicrobial effects of MB and Eosin Methylene blue (EMB) agar against colistin-resistant AB strains. Methods The AB ATCC 19606 strain and 31 AB clinical isolates were included in the study. In the first round, ATCC strain and a clinical isolate were transformed into colistin-resistant forms, using Li's method, with increasing colistin concentrations. At each step, new MICs were determined and subcultures were inoculated to EMB and sheep blood agar (SBA). The colistin MIC values of the subcultures were also determined using Mueller Hinton Agar (MHA) containing 14 µg/mL MB. In the second round, colistin resistant clones of all collected clinical isolates (n=31) were obtained and screened to investigate their susceptibility to EMB agar by inoculating on SBA and EMB agar. Results At the beginning, the MICs of two strains were 0.5 µg/mL. At the last stage, both MICs had risen to 64 µg/mL. Subpopulations with high colistin resistance (≥ 32 µg/mL) were inhibited by MB and EMB agar, but could grow well on SBA. In MHA plates containing MB, the MICs decreased to the 0.5 µg/mL level for colistin-susceptible or moderately resistant clones. Additionally, clones with high colistin resistance showed atypical colony morphology on SBA. In the second round, MICs of the colistin resistant clones of all clinical isolates rose to 8 µg/mL after colistin exposure and 35% of those clinical isolates were inhibited by EMB agar while they could grow on SBA. Conclusion Highly resistant strains were totally inhibited by the effect of MB and EMB agar, while the MICs of the susceptible and moderate resistant clones decreased. EMB agar and MB may have inhibitory effects against colistin-resistant AB strains and MB may have a potential to be used as an antimicrobial drug. Secondly, using only EMB agar for subculturing may cause missing of colistin-resistant strains and giving incorrect identification or antibiogram reports in clinical microbiology laboratories.

Background

Acinetobacters are non-fermenting Gram-negative opportunistic pathogens causing infections in the respiratory tract, urinary tract, and wounds; they also cause septicemia [1]. *Acinetobacter baumannii* (AB) is a major hospital-associated pathogen that causes a spectrum of diseases including infections of the respiratory tract, bloodstream, urinary tract, surgical sites, and wounds [2]. AB has a propensity to acquire resistance to multiple classes of antimicrobial agents, and treatment of infection by highly resistant strains can be extremely difficult [3, 4].

Colistin is a last resort antimicrobial drug that can be chosen for treatment of patients infected with carbapenem-resistant *Acinetobacter baumannii* and combination therapies with colistin are recommended to overcome these infections [5-7]. However, in recent years, heteroresistance and resistance to colistin have been reported for AB [8-10]. The colistin-resistant AB rate was found to be as high as 40.6 % in a study in Spain [11]. Oikonomou et al. reported that the colistin resistance rate of AB had increased and had reached 21.1 % in Greece in 2015 [12]. Queenan et al. performed a surveillance

study of US hospitals and reported that 5.3% of all *Acinetobacter* strains were resistant to colistin [13]. Various studies from clinical settings throughout the world have indicated the emergence of colistin-resistant AB as an important healthcare problem [10, 14]. Colistin-susceptible AB strains can easily transform into colistin-resistant or heteroresistant clones, or colistin heteroresistant strains can transform into colistin-resistant clones via exposure to colistin [15-18]. In 2016, the emergence of plasmid-mediated colistin resistance in an *E. coli* strain by the MCR-1 mechanism was reported from China, which made scientists focus on the urgent need for global action in the fight against pan-drug-resistant Gram-negative bacteria [19].

Methylene blue (MB), also known as methylthioninium chloride, is the major compound of EMB agar, which is also a medication and microbiological dye [20, 21]. In the past, it has been used as an antidote for cyanide poisoning [22]. It is used in microbiological science for bacterial staining, in internal medicine as an antidote to drug-induced methemoglobinemia, ifosfamide-induced encephalopathy, and in surgical sciences for staining of the surgical field [22-27]. It was reported to show activity against *Plasmodium falciparum* strains and to have the potential to be used as an antimalarial agent in combination with other drugs [28]. Experts recommend a maximum total dose of 5-7 mg/kg for treatment of drug-induced methemoglobinemia during the first few hours of treatment [29]. Reports suggest it can be administered by intravenous infusion at varying times during parathyroid surgery at doses ranging from 3 mg/kg to 7.5 mg/kg [30].

During our previous study investigating the inhibitory effects of antibiotic combinations on colistin resistance, we observed that AB strains that had developed high level colistin resistance could not grow on eosin methylene blue (EMB) agar [18]. EMB agar is commonly used to culture Gram-negative microorganisms in clinical microbiology laboratories. The ingredients of EMB agar are peptone, lactose, K₂HPO₄, eosin yellowish, MB (0,065 g/L) and agar-agar [20, 31]. Since MB is one of the two major ingredients of EMB agar along with eosin, and it can also be used as a drug in medicine, we aimed to investigate the antimicrobial effects of MB aqueous solution on AB clones that had developed colistin resistance. At the same time, we also investigated the inhibitory effects of EMB agar on such clones. In this study, we did not test the activity of eosin compound since it is a hazardous chemical and has no potential to be used as a medication in clinical practice. If inhibitory effect of MB on colistin resistant AB strains can be proven, a new drug containing MB and killing this superbug could be developed in the future.

Methods

An AB American Type Culture Collection (ATCC) 19606 standard strain and 31 colistin-susceptible multi drug-resistant AB clinical isolates were included in the study. The isolates were obtained from the blood cultures of 25 patients in the general intensive care unit of Çanakkale Onsekiz Mart University Hospital during a study period from January 2010 to September 2012. The AB ATCC 19606 strain was obtained from Çanakkale Onsekiz Mart University Clinical Microbiology Department collection.

The isolates were identified as AB using the VITEK2 identification system (Biomeriux, France) and antibiograms were performed by this system. The results were evaluated according to Clinical Laboratories Standards Institute (CLSI 2017) criteria [32]. The strains were stored at -80 °C in a brain heart infusion broth containing 15% glycerol. Before the study, the AB ATCC 19606 and the stored AB isolates were subcultured on sheep blood agar twice and then they were incubated for 24 hours at 37 °C in an incubator.

For imipenem and meropenem, antimicrobial resistance profiles were also confirmed by using Mueller Hinton Agar (MHA) and gradient antimicrobial test strips (MIC evaluator strips, Oxoid, UK). The tests were performed and evaluated according to the manufacturer's recommendations and CLSI criteria [32]. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality control [32]. For colistin, the antimicrobial susceptibilities of AB ATCC 19606 and the clinical isolates were also determined by gradient test and confirmed by a micro-broth dilution test using Cathion Adjusted Mueller Hinton Broth (CAMHB) and colistin sulfate water-soluble powder (Sigma, USA). The results were evaluated according to CLSI criteria [32]. *Paeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality control [32].

In the first round, only the AB ATCC 19606 strain and one selected colistin susceptible clinical isolate (strain no: 1) were included. In this round, we wanted to observe the methylene blue and EMB agar susceptibilities of the same clones with different MIC levels. So, serial dilution method of Jian Li was used to obtain colistin-resistant AB clones from the parent strains as below [8]. Step by step, two strains were transformed to colistin-resistant forms by colistin exposure in broth media at increasing colistin concentrations over nine days. In each step the MIC values of the strains were elevated. Afterwards, effects of methylene blue and EMB agar on the clones with new and higher MICs were analyzed.

On day 1, 10 mL of 2 CAMHB media containing colistin sulfate at 0.5x MIC (equal to 0.25 µg/mL) were separately inoculated with the ATCC strain and clinical isolate from three fresh colonies (Passage 1). The cultures were incubated at 37 °C in a shaking water bath (100 rpm) for 48 hours [8].

On day 3, 0.1 mL of the culture was transferred into 10 mL of CAMHB containing colistin sulfate at 2x MIC (Passage 2), and the cultures were incubated for 48 hours (see above).

On day 5, 0.1 mL of the culture of AB from passage 2 was transferred into 10 mL of CAMHB containing colistin sulfate at 32x MIC (Passage 3), and the cultures were incubated for 48 hours [8].

On day 7, 0.1 mL of the culture of AB from passage 3 was transferred into 10 mL of CAMHB containing colistin sulfate at 64x MIC (Passage 4), and the cultures were incubated for 48 hours [8].

After each passage and incubation (Passages 1, 2, 3, 4), 50 microliters of the culture were taken from the CAMHB test tube and inoculated onto Sheep Blood agar (SBA) and Eosin Methylene Blue agar (Salubris, Turkey). Additionally, in the last three steps (Passages 2, 3, 4), the cultures were also inoculated onto MHA plates containing 14 µg/mL of methylene blue powder (Zag, Turkey). After each step, the new

colistin MIC values of the subcultures were determined from colonies on SBA by using a gradient test and a broth microdilution test (gold standard for colistin) [32].

In the last step (Passage 4), two different colony types appearing/growing on SBA were inoculated onto 2 SBA plates for purification. After purification, each colony type was studied as above in order to analyze their EMB and MB sensitivities and the colistin MIC values. Both colony types were also identified by the Matrix Assisted Laser Desorption/Ionization (MALDI) time of flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) using the MALDI Biotyper software package (version 3.0) in order to confirm the identification and exclude the risk of contamination.

In the second round, colistin resistant clones which were obtained from 31 multi drug-resistant and colistin susceptible AB clinical isolates were screened to investigate their susceptibilities to EMB agar. All 31 colistin susceptible parent isolates were transformed to colistin resistant clones by sub-inhibitory exposure to colistin as following. Three fresh colonies from each isolate were inoculated into 10 mL of CAMHB tube containing 0.25 µg/mL colistin and all tubes were incubated for 48 hours at 37 °C in a shaking water bath. After this procedure MHA plates containing 4 µg/mL colistin were inoculated with 0.1 mL of the culture from CAMHB tubes and incubated for 48 hours at 37 °C in an incubator. In this step, we aimed to select and isolate colistin resistant AB sub-populations from the bacterial suspensions. The colonies those could grow on MHA after incubation were considered to be resistant to colistin and subcultured on SBA and EMB agar. After 24 hours incubation at 37 °C, the growing rates of all 31 clones on SBA and EMB agar were analyzed. In addition, new colistin MIC values for these clones were determined and confirmed to be >4 µg/mL using the broth microdilution method (from the fresh colonies on SBA).

Results

Antibiogram results of parent isolates and standard strain:

The clinical isolates were resistant to ampicillin/sulbactam, piperacillin, ceftazidime, cefoperazone/sulbactam, cefepime, imipenem, meropenem, ciprofloxacin, tetracycline and trimethoprim/sulfamethoxazole, and had intermediate resistance to amikacin, and were susceptible to gentamicin and colistin by the VITEK2 antibiogram system. Carbapenem resistance of the clinical isolates were confirmed with MIC evaluator strips and the isolates were found resistant to imipenem and meropenem with MIC > 32 µg/mL.

The standard ATCC strain was resistant to trimethoprim/sulfamethoxazole, had intermediate resistance resistant to cefepime, and was susceptible to ampicillin/sulbactam, piperacillin, ceftazidime, cefoperazone/sulbactam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tetracycline and colistin by the VITEK2 antibiogram system. Imipenem and meropenem susceptibilities were also confirmed with MIC evaluator strips and it was found that MIC = 0.25 µg/mL for both carbapenems.

Colistin MIC values for the clinical isolates and ATCC strain were also determined by using a gradient test. MICs for the clinical isolates were found between 0.5 and 0.75 µg/mL and MIC for the ATCC strain was found 0.5 µg/mL. Colistin MIC values for the clinical isolates and ATCC strain were confirmed using the broth microdilution method. All were found susceptible to colistin with MIC = 0.5 µg/mL.

New MIC results of two strains during serial dilution test in the first round:

At the beginning, the colistin MICs for the selected clinical isolate (no:1) and ATCC strain were 0.75 µg/mL and 0.5 µg/mL, respectively (by the gradient test). After 48 hours incubation with 0.5x MIC colistin concentrations, the MIC values of the strains rose to 0.75 µg/mL and 1 µg/mL, respectively (*step 1*). After 48 hours incubation with 2x MIC colistin concentrations, the colistin MIC values of the strains rose to 1 µg/mL and 3 µg/mL, respectively (*step 2*). After 48 hours incubation with 32x MIC colistin concentrations, the new MIC values of the strains rose to the 32 µg/mL level for both strains (*step 3*). But in this step, interestingly, the clone of the clinical isolate became heteroresistant and there was another inhibition zone with MIC=2 µg/mL. Therefore, we determined two different MICs for clinical isolate in this step (Outer zone MIC=32 µg/mL, inner zone MIC=2 µg/mL) (*step 3*). In *step 4*, the MIC of the clinical isolate increased to 64 µg/mL level and no heteroresistance was observed this time. However, regarding the ATCC clone, we observed two different colony morphologies (large-mucoid and small-pale) on SBA and decided to determine the MIC values of each colony types separately. For this reason, we purified two different colony types by subculturing a single colony onto another SBA. After the purification step, we determined that the MIC of the large-mucoid colony type was 1 µg/mL, but the MIC of the small-pale colony type was 64 µg/mL. So we had another heteroresistant clone in this step. The new MIC results obtained during the serial dilution test are summarized in Table 1.

For determination of the new colistin MICs, the clones (with elevated colistin MICs) were inoculated to normal MHA plates and MHA plates containing 14 µg/mL MB powder (MBMHA) in steps 2, 3 and 4.

In step 2, the colistin MIC values of the ATCC strain and the clinical isolate were found to be 1 µg/mL and 3 µg/mL, respectively (on normal MHA). But their MIC values decreased to 0.5 µg/mL level on MBMHA.

In step 3, the MIC value of the ATCC strain was 32 µg/mL on MHA, but on MBMHA, the colistin-resistant bacteria were totally inhibited by MB and no growth was observed (Figure 1A, 1B). For the heteroresistant clinical isolate (MIC: 32, 2 µg/mL), the MIC value decreased to the 0.5 µg/mL level on MBMHA by the effect of MB (Figure 1C, 1D).

In step 4, the ATCC strain exhibited two different colony morphologies on SBA. The first colony type was small-pale, the second colony type was large-mucoid (Figure 2A). Each type of ATCC colony was investigated after being subcultured onto another SBA (Figure 3). The MIC value of the small-pale colony type was found 64 µg/mL on MHA, but on MBMHA, the colistin-resistant clones were totally inhibited by the effect of MB and no growth was observed. The MIC value of the large-mucoid colony type was found to be 1 µg/ml on MHA, but decreased to 0.5 µg/mL on MBMHA (Table 1).

In step 4, the clinical isolate colonies appeared homogeneously small-pale on SBA. The MIC value was 64 µg/mL on MHA, but on MBMHA, the colistin-resistant bacteria were totally inhibited by MB and no growth was observed. The results are summarized in Table 1.

EMB agar sensitivities of two strains with elevated MICs in the first round (Growth characteristics on EMB agar and SBA)

Subcultures of the clinical isolate and ATCC strain could grow on both SBA and EMB agar in steps 1 and 2 (in these steps all the MIC values of the clones were ≤ 3 µg/mL).

In step 3, we observed only small-pale colonies (MIC= 32 µg/mL) on the SBA plate of the ATCC strain. When we examined the SBA plate of the clinical isolate, we detected two types of colony (small-pale, large-mucoid). The small-pale colonies of both strains were totally inhibited on EMB agar while the large-mucoid colonies could survive on EMB agar (Table 2, Figure 2A, 2B, 2C, 2D).

In step 4, we observed only small-pale colonies (MIC= 64 µg/mL) on the SBA plate of the clinical isolate (Figure 2C) and they were totally inhibited on the EMB agar plates (Figure 2D). On the SBA plate of the ATCC strain, we detected two types of colony, which were small-pale and large-mucoid (Figure 2A). Small-pale colonies were totally inhibited by EMB while large-mucoid colonies could survive on EMB agar (Figure 2B). As a fifth step, two different colony types from SBA were also subcultured onto another SBA and EMB agar for confirmation (Figure 3). We again determined that the small-pale colony type was inhibited by EMB while the large-mucoid colony type could grow on both SBA and EMB agar. All results are summarized in Table 2.

After the induction and selection procedure of resistant clones, new MIC values of all 31 clinical isolates rose to 8 µg/mL. At the end of the EMB sensitivity/screening test, 11 out of 31 resistant clones could not grow on EMB agar (35 %) while they could grow well on SBA.

Discussion

Colistin has been progressively used as a last chance therapy for severe infections in critically ill patients [33]. It is bactericidal against Gram-negative bacteria, and its amphiphilic nature allows it to interact with lipid A moiety of lipopolysaccharide (LPS) causing disarray in the bacterial outer membrane [34]. According to the first hypothesis, the colistin resistance is mediated by loss of LPS production, caused by mutations in any of the lipid A biosynthesis genes (*lpxA*, *lpxC* and *lpxD*) terminating complete production of LPS [35]. LPS deficiency causes less negative charge and thus might be the reason for the loss of affinity toward colistin [36]. Secondly, colistin resistance has been hypothesized to *PmrAB*-two component response regulator and sensor kinase system affecting the expression of genes implicated in lipid A modification and thus causing colistin resistance [37]. Qureshi et al. reported that colistin-resistant AB occurred almost exclusively among patients who had received colistin for treatment of carbapenem-resistant, colistin-susceptible AB infection, and observed that colistin resistance in hospital settings is strongly associated with lipid A modification by phosphoethanolamine [38]. In their study, Beceiro et al.

hypothesized that the *pmrAB* resistance mechanism could be observed in patients treated with colistin [39].

It has been shown that acquiring colistin resistance may result in increased susceptibility to several antibiotics including amikacin, gentamicin, azitromycin, rifampicin and vancomycin [40-42]. Also in one of our studies we reported the emergence of *in vitro* vancomycin susceptibility in AB clones that had developed colistin resistance [43]. It was also suggested that mutations in the *pmrAB* mechanism are more likely to have a cost in terms of overall fitness and the virulence of the bacterial strain, and increase susceptibility to several antibiotics apart from the *lpxACD* mechanism [12, 41, 42]. In accordance with previous studies, we observed in this study that colistin-resistant AB populations grew weaker on media in comparison to colistin-susceptible populations [39, 41, 42].

Similar to these findings, we observed in our study that clones that had developed colistin resistance acquired susceptibility to MB and could not grow on EMB agar. In step 2, we observed a decrease in the colistin resistance levels of both strains growing on MHA plates containing MB. In this step, the resistance level decreased from 1 µg/mL to 0.5 µg/mL for the ATCC strain and from 3 µg/mL to 0.5 µg/mL for the clinical strain (Table 1). We hypothesized that, in this step, MB selectively inhibited growth of the subpopulations having colistin MIC values ≥ 1 µg/mL. Since 1 and 3 µg/mL were not very high MIC values and close to 0.5 µg/mL, there might be some subpopulations with 0.5 µg/mL that were not affected by MB and that continued growing in the presence of MB. As a result, MB selectively killed AB clones having MICs ≥ 1 µg/mL. In accordance with this hypothesis, both clones were not inhibited by EMB agar in this step.

In steps 3 and 4, AB populations having high levels of colistin resistance (MIC = 32 µg/mL and 64 µg/mL) were totally inhibited by MB and could not grow on EMB agar plates. On the other hand, the colistin heterosensitive subpopulations with lower MICs in steps 3 and 4 (MIC = 1 µg/mL and 2 µg/mL) were not inhibited by MB but their MICs decreased to 0.5 µg/mL by MB exposure in accordance with step 1. In steps 3 and 4, the small colony type was inhibited on EMB agar while both large and small colony types could grow on SBA. In step 4, we purified small-pale and large-mucoid colony types on another SBA (Figure 3.) and we found that the MIC of the small-pale colony type was 64 µg/mL while the MIC of the large-mucoid colony type was 1 µg/mL. In our study, large-mucoid colony types were not inhibited on EMB agar (Figure 2A, 2B), so this case confirmed our observation that large AB colonies have lower MICs compared to small colonies.

In the EMB screening round (2nd round), only 35% of the clinical isolates were inhibited by EMB. We thought that this inhibition is caused by MB compound of the medium. However, 65 % of isolates were not inhibited in contrast to our expectation. Since 8 µg/mL is not a very high MIC level for colistin, EMB or MB could have failed to kill some mutant or colistin susceptible subpopulations (we observed that there could be a relation between colistin MIC level and killing effect of EMB/MB).

In the light of these findings, we hypothesize that MB inhibits AB subpopulations with colistin MIC >1 µg/mL. When the colistin MIC value of the AB strain is relatively low, MB only increases the susceptibility of the strain and decreases the MIC value to 0.5 µg/mL by inhibiting subpopulations with higher MIC levels. But when the MIC of the strain is very high, the numbers of colistin-susceptible subpopulations are very low and MB totally inhibits growing of this strain.

Conclusions

In conclusion, our research has three important outcomes. First MB inhibits the growth of AB strains with high level colistin resistance or increases the susceptibility of AB strains with lower MICs to colistin. Thus, MB might have a potential to be used as a drug for colistin-resistant AB or to increase the colistin susceptibility of AB strains. There are several reports that have investigated the effects of MB on AB strains but these reports were not focused on colistin-resistant AB strains or they evaluated the effects of MB upon irradiation with a light source (photodynamic therapy) [44, 45]. MB may be a promising agent during outbreaks of colistin-resistant AB. It can be administered by an intravenous route or can be used to decontaminate hospital environments and especially ventilator systems in intensive care units. After the study, we applied to the European Patent Office for an indication patent to keep rights to use MB as a drug to treat colistin resistant AB infections [46]. We hope that MB could be used as an antimicrobial agent to treat infections caused by colistin resistant AB in the future.

Second outcome; EMB agar is one of the most common media used in microbiology laboratories for Gram-negative bacteria plating. It is used in both clinical microbiology and scientific research laboratories [20, 21]. Thus, using only EMB agar in clinical laboratories may cause colistin-resistant AB species to be absent or may result in determining lower colistin MIC values (false susceptible) after the antibiogram procedure. So, diagnosis and antimicrobial treatment of patients may be effected in this case. In the light of our findings, EMB agar should not be used as a subculturing media for AB isolates in hospital laboratories.

As a third point; we detected two different colony variants of AB: the small-pale colony type with high MICs to colistin, and the large-mucoid colony type with low colistin MICs. Since the small-pale colony type appears more like a Gram positive bacterial species under macroscopic examination, it might be confusing and difficult for a clinical microbiologist to identify this atypical AB variant. Furthermore, clinical microbiologists should be alert if they isolate a small-pale AB colony variant because of its potential to display a high level of colistin resistance.

Beyond all these outcomes, our study has some limitations that should be mentioned. It was an *in vitro* study and MB susceptibility development needs to be confirmed by using more colistin-resistant AB clinical isolates. Secondly, we could not perform a molecular study to understand the specific colistin resistance development mechanism of MB-susceptible AB strains in our research. Since the *pmr* system is claimed to be much more associated with clinical colistin resistance [12, 39], in a second study, the colistin resistance mechanism of MB-susceptible AB clones could be investigated before recommending

MB as a new drug. In addition, further molecular and genetic analyses are necessary to understand how MB susceptibility develops in colistin-resistant AB strains.

Declarations

Ethics approval and consent to participate: Ethical approval has been obtained for the study from the Institutional Clinical Research Ethics Committee of Çanakkale Onsekiz Mart University (Faculty of Medicine) with the number 18920478-050.01.04/E.19725 (23.02.2016).

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: DG has conceptualized and designed the study. He has contributed to drafting the manuscript, revising for important intellectual content, analysis and interpretation of data; and given final approval to the version to be published. MTO and AA has made contribution in acquisition of data, analysis and interpretation of data and preparing the text of manuscript. All authors take responsibility for all portions of the content. All authors read and approved the final manuscript.

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Tables

Table 1. MIC results during serial dilution test in the first round (in µg/mL)

	ATTC STRAIN	CLINICAL ISOLATE
ORIGIN	MIC= 0.5	MIC= 0.75
STEP 1	MIC= 0.75	MIC= 1
STEP 2	MIC= 1 MIC on MBMHA= 0.5	MIC= 3 MIC on MBMHA= 0.5
STEP 3 (Fig 1.)	MIC= 32 MIC on MBMHA: No growth	MIC: Double (heteroresistant) Inner zone: 32 Outer zone: 2 MIC on MBMHA= 0.5
STEP 4	-Two types of colonies grew on SBA (Large and small); -Two different colony types were subcultured onto SBA to analyze specific characteristics (Fig 2.) Small colony: MIC= 64 MIC on MBMHA: No growth Large colony: MIC= 1 MIC on MBMHA= 0.5	-One type of colony grew on SBA (only small) (Fig 2.) Small colony: MIC= 64 MIC on MBMHA: No growth

* MIC: Minimum inhibitory concentration, SBA: Sheep blood agar, MBMHA: Mueller Hinton agar with methylene blue.

Methylene blue sensitivities of two strains with elevated MICs in the first round

Table 2. Growth characteristics of the two strains on EMB agar and SBA

	ATTC STRAIN	CLINICAL ISOLATE
ORIGIN	SBA:+	SBA:+
	EMB:+	EMB:+
STEP 1	SBA:+	SBA:+
	EMB:+	EMB:+
STEP 2	SBA:+	SBA:+
	EMB:+	EMB:+
STEP 3	SBA:+ Only small colonies	SBA:+ Large and small colonies
	<u>EMB: Ø</u>	EMB:+ Only large colonies
STEP 4	SBA:+ Large and small colonies	SBA:+ Only small colonies
	(Figure 2.) EMB:+ Only large colonies	<u>EMB: Ø</u>

Subculture from a single large type of colony:

SBA:+

EMB:+

Subculture from a single small type of colony:

SBA:+

EMB: Ø

* SBA: Sheep blood agar, EMB: Eosin methylene blue agar, +: positive growth, Ø: no growth

MIC values and EMB agar screening results of the clinical isolates in the second round

Figures

Figure 1

Antibiograms of subcultures on MHA and MBMHA at Step 3

Figure 2

Growth characteristics of subcultures on EMB agar and SBA at Step 4

Figure 3

Appearances of two different colony types of *A. baumannii* Footnotes for Figure 3A. Large-mucoid colony morphology on SBA Footnotes for Figure 3B. Small-pale colony morphology on SBA