

A Simple Method for Assessment of MDR Bacteria for Over-Expressed Efflux Pumps

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Abstract: It is known that bacteria showing a multi-drug resistance phenotype use several mechanisms to overcome the action of antibiotics. As a result, this phenotype can be a result of several mechanisms or a combination of thereof. The main mechanisms of antibiotic resistance are: mutations in target genes (such as DNA gyrase and topoisomerase IV); over-expression of efflux pumps; changes in the cell envelope; down regulation of membrane porins, and modified lipopolysaccharide component of the outer cell membrane (in the case of Gram-negative bacteria). In addition, adaptation to the environment, such as quorum sensing and biofilm formation can also contribute to bacterial persistence. Due to the rapid emergence and spread of bacterial isolates showing resistance to several classes of antibiotics, methods that can rapidly and efficiently identify isolates whose resistance is due to active efflux have been developed. However, there is still a need for faster and more accurate methodologies. Conventional methods that evaluate bacterial efflux pump activity in liquid systems are available. However, these methods usually use common efflux pump substrates, such as ethidium bromide or radioactive antibiotics and therefore, require specialized instrumentation, which is not available in all laboratories.

In this review, we will report the results obtained with the Ethidium Bromide-agar Cartwheel method. This is an easy, instrument-free, agar based method that has been modified to afford the simultaneous evaluation of as many as twelve bacterial strains. Due to its simplicity it can be applied to large collections of bacteria to rapidly screen for multi-drug resistant isolates that show an over-expression of their efflux systems. The principle of the method is simple and relies on the ability of the bacteria to expel a fluorescent molecule that is substrate for most efflux pumps, ethidium bromide. In this approach, the higher the concentration of ethidium bromide required to produce fluorescence of the bacterial mass, the greater the efflux capacity of the bacterial cells. We have tested and applied this method to a large number of Gram-positive and Gram-negative bacteria to detect efflux activity among these multi-drug resistant isolates. The presumptive efflux activity detected by the Ethidium Bromide-agar Cartwheel method was subsequently confirmed by the determination of the minimum inhibitory concentration for several antibiotics in the presence and absence of known efflux pump inhibitors.

Keywords: Clinical isolates; Efflux activity; Efflux pumps; Ethidium bromide; Multi-drug resistance; Screening method.

EXISTING METHODS TO ASSESS BACTERIAL EFFLUX ACTIVITY

The increasing multi-drug resistance reported in bacterial clinical isolates has become a major concern in the public

health area given that the lack of treatment options for these infections, with the existing antibiotics, is becoming more and more problematic [1-3]. As multi-drug resistance can be the result of an over-expression of the bacterial efflux pump systems, used to extrude unrelated antibiotics prior to their reaching the intended targets [3], there is a need to develop and implement new and improved methods for a real-time and quick identification of efflux mediated multi-drug resistant (MDR) phenotypes [4, 5]. Primarily, assessment of efflux activity has been conducted using a common substrate of efflux pumps, such as ethidium bromide (EtBr). This

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compound is widely used to “monitor” efflux in a given bacteria and its increased retention after an uncoupler of the proton motive force or other efflux inhibitor has been added [4-6]. The baseline fluorescence or the increase of EtBr that is retained by the bacteria prior to and after the addition of the uncoupler (or other type of inhibitor), is usually determined using specialized and expensive fluorometric instrumentation that may not be available in a clinical bacteriology laboratory [5, 7]. The principle of these fluorometric assays is the passage of EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell [8]. EtBr traverses the bacterial cell wall (in the case of Gram-negative bacteria *via* porin channels) and once inside, it can be concentrated to a point where it fluoresces when excited by ultraviolet (U.V.) light. Efflux pumps of MDR bacteria recognize this substrate and are able to extrude it to the medium [6, 8]. These efflux systems are temperature dependent [9] and this process will continue if the concentration of EtBr in the culture medium does not overcome the capacity of the bacterial efflux pump itself. Therefore, loading of the bacteria with EtBr has to take place at a concentration that is well below its minimum inhibitory concentration (MIC) [4, 5]. This way, the signal that is generated can be continuously recorded under specific conditions, such as time, temperature and different properties of the medium (for example: pH; calcium availability; *etc.*) [5, 6]. Efflux of EtBr can be readily shown by the use of standard fluorometers or cytometers [7, 10, 11]. However, when efflux is due to over-expressed efflux pumps, it cannot be easily shown/assessed by some of the conventional methods previously referred to [5, 12]. The current fluorometric systems used to access efflux activity are in general non-physiological; usually the control of the temperature is restricted; the methods are cumbersome and do not yield data that can be subjected to standardization for intra-laboratory comparison. Moreover, due to the wide variety of instrumentation, reagents, media, *etc.*, used by the many laboratories working in this area, it is difficult to compare and standardize the results obtained from these studies [4, 5]. In the recent years there have been several methods published that can assess efflux in a more dynamic and physiological way making use of fluorescent probes, such as EtBr, Hoechst-33342 (bisbenzimidazole) or fluorogenic compounds (for example: Fluorescein-di- β -D-galactopyranoside) or using lipophilic dyes, such as Nile Red [4, 5, 6, 12-15]. The majority of these methods use a liquid system and need equipment that may or may not be available in all the clinical laboratories. Therefore, there is still an obvious need to establish new and optimized assays for the assessment of efflux pumps of distinct bacteria, in particular those of clinical origin showing an MDR phenotype. Additionally, there is still a lack of methods that make use of a solid system and simple equipments. A new assay should be simple, quick to perform, reproducible and should excuse any specialized instrumentation for its conductance [16]. In MDR bacteria, the assessment of efflux pump activity can help to characterize the basis by which novel/existing compounds inhibit efflux activity, consequently rendering the organism susceptible to one or several of the antibiotics to which it was initially resistant [8, 17-19]. If those compounds were available for clinical therapy, it would be expected that the assay would have extensive implementation within the hospital/clinic and provide much of the guidance

needed for the administration of the compounds to patients infected with an MDR bacteria [19]. Moreover, the availability of such compounds brings benefits to the therapeutic regimens, resulting in the opportunity to use outdated, inexpensive and safe antibiotics that had been made redundant as a consequence of the emergence of MDR bacteria [20]. To describe and characterize in detail the activity of an efflux inhibitor, the method by which that activity is defined and quantified must be reliable and feasible to apply to a large number of inocula, yielding inter-laboratory standardization.

ETHIDIUM BROMIDE (ETBR)-AGAR CARTWHEEL METHOD

This simple, instrument-free, agar-based method utilizes EtBr for the demonstration of efflux pump activity in bacteria [21]. It is an improved and optimized version of the previously described EtBr-agar method [16]. This new optimized method can be applied simultaneously to up to twelve bacterial strains to identify clinical isolates that have an over-expressed efflux activity [21]. Each plate should include at least one reference strain that will serve as a comparative control for fluorescence analysis. However, the number of reference strains can be increased to two or more, depending on a given experiment. In addition, it allows the evaluation of agents that may inhibit this same activity. The effect of temperature on the efflux pump system can also be assessed/confirmed. The methodology used is straight-forward employing the preparation of two sets of Trypticase Soy Agar (TSA) plates containing EtBr concentrations ranging from 0.0 to 2.5 mg/L (these concentrations may vary according to the bacterial strain in study). The TSA plates should be prepared fresh on the previous or same day of the experiment and kept protected from light. Overnight cultures of the bacterial isolates to be tested are prepared in liquid media and in the following day their concentration adjusted to 0.5 of a McFarland standard. The TSA plates are then divided into as many as twelve sectors by radial lines, forming a cartwheel pattern (Fig. 1). The adjusted bacterial cultures are then swabbed on the EtBr-TSA plates starting from the center of the plate to the margin. The TSA plates are then incubated at 37°C for 16 hours. After this period, the TSA plates are examined under a gel-imaging system (or a U.V. transilluminator); the minimum concentration of EtBr that produces fluorescence of the bacterial mass recorded; and the TSA plates photographed (Fig. 1).

In the case that one wants to check for the temperature effect, then an additional incubation step should be performed. In this case, one of the TSA plate sets should be re-incubated at 37°C, whereas the duplicate set is transferred to 4°C. At the end of an additional 24 hours period, the TSA plates are observed and photographed again and the minimal concentration of EtBr that produced fluorescence at each temperature will be compared to that evident after the first incubation (at 37°C).

TESTING OF THE METHOD IN CLINICALLY RELEVANT MDR GRAM-NEGATIVE BACTERIA

All the clinical isolates evaluated by the EtBr-agar cartwheel method and inserted in this study have a confirmed MDR phenotype. This MDR was defined as resistance to

the other strains that didn't show fluorescence or showed lower fluorescence than the reference strains (data not shown).

[EtBr] (mg/L)	0	0.25	0.5	1	1.5	2
37°C						
4°C						

Fig. (4). The effect of the temperature in the efflux activity of *Acinetobacter* strains screened by the EtBr-agar cartwheel method. The strains were streaked in the EtBr-TSA plates following the following scheme:

ACI – *Acinetobacter* clinical isolate.

The effect of the temperature on the efflux activity of the bacterial strains can be assessed by the EtBr-agar cartwheel method. As an example, a set of *Acinetobacter* strains is illustrated in the figure. As visible, after a period of incubation at 4°C, strains that didn't show fluorescence at 37°C are highly fluorescent at the higher concentrations of EtBr tested in the TSA plate.

Table 1. The Effect of Selected EPIs on the Resistance of the Induced and MDR Gram-negative Bacteria

Species	Strain	MIC (mg/L)							
		TET				CIP			
		-	+ TZ	+ CPZ	+ PAN	-	+ TZ	+ CPZ	+ PAN
E. coli ^a	AG100	1	0.5	0.5	0.5	0.015	0.003 (5x)	0.003 (5x)	0.0075
	AG100 _{TET}	12	0.375 (32x)	3 (4x)	3 (4x)	0.06	0.015 (4x)	0.03	0.03
	HEMEC7	128	32 (4x)	16 (8x)	32 (4x)	0.25	0.125	0.125	0.125
	HEMEC10	64	32	16 (4x)	32	16	8	4 (4x)	2 (8x)
Enterobacter aerogenes ^a	ATCC15038	TET				NOR			
		-	+ TZ	+ CPZ	+ PAN	-	+ TZ	+ CPZ	+ PAN
	HMEA11	2	1	0.125 (8x)	1	0.03	0.007 (4x)	0.003 (10x)	0.015
Salmonella ^a	NCTC13349	TET				CIP			
		-	+ TZ	+ CPZ	+ PAN	-	+ TZ	+ CPZ	+ PAN
	5408 _{CIP}	1	0.25 (4x)	0.5	0.5	32	8 (4x)	16	8 (4x)
Acinetobacter	ATCC 19606	MER				IMI			
		-	+ TZ	+ CPZ	+ PAN	-	+ TZ	+ CPZ	+ PAN
	ACI7	128	64 (2x)	0.25 (9x)	16 (3x)	≥ 256	128	128	≥ 256
	ACI3	128	128	128	64 (2x)	≥ 256	128	128	≥ 256

TET - tetracycline; CIP - ciprofloxacin; NOR - norfloxacin; MER – meropenem; IMI – imipenem; TZ - thioridazine; CPZ - chlorpromazine; PAN - Phe-Arg-naphthylamide. ^apartial data previously published in [21]. Solutions of TZ, CPZ and PAN (Sigma Aldrich, SA, Madrid, Spain) were prepared in deionized water. Aliquots of stock solutions of the phenothiazines CPZ and TZ were protected from light and stored at -20°C. Working solutions were prepared on the day of the experiment. To assure that the efflux inhibitors do not compromise the bacterial viability, each compound was employed at a concentration of 1/2 of the MIC. Exceptions to this was PAN, known to affect the cellular viability even at 1/2 of the MIC [30] and in this case the compound was used at a maximum of 20 mg/L (value well below their pre-determined MIC). Values in bold-type correspond to a decrease of 4-fold or higher on the MIC values in comparison to the ones in the absence of inhibitor. Values in parenthesis indicate the MIC decrease relative to the one of the original culture. A reduction in the MIC of at least one-fourth of their original values in the presence of the efflux inhibitor was considered indicative of the presence of efflux activity. All assays were performed in triplicate.

CONFIRMATION OF THE RESULTS PROVIDED BY THE ETBR-AGAR CARTWHEEL METHOD

The results provided by the EtBr-agar cartwheel method were further explored by the additional determination of the MIC values for selected antibiotics, known to be efflux pump substrates (e.g. tetracycline, norfloxacin, imipenem, etc.), in the presence of compounds described as having an inhibitory effect on efflux activity. These compounds can include a variety of classes, such as efflux inhibitors, permeabilizers, chemosensitizers, etc. Ultimately, the intended use of these compounds is to confirm the activity of over-expressed efflux system(s) in the MDR clinical strains identified by this method. This confirmation is important to rule out other factors that may affect fluorescence such as a decrease in the cellular permeability to EtBr [6, 8, 28, 29]. Isolates that fluoresced at concentrations of EtBr significantly greater than their reference strains were evaluated for their susceptibility to a panel of antibiotics known to be substrates of efflux pump systems described for each bacterial species, in the presence and/or absence of compounds known to inhibit efflux activity. For comparative terms, representative strains showing fluorescence at low EtBr concentrations were also evaluated (Tables 1 and 2).

The efflux inhibitors used for Gram-negative and Gram-positive bacteria can vary but some extensively studied compounds are usually used such as: thioridazine (TZ), chlorpromazine (CPZ), Phenylalanine-Arginine beta-Naphthylamide (PAN) (in the case of Gram-negative bacteria) and

TZ, CPZ and reserpine (RES) (in the case of Gram-positive bacteria) Table 3. To assure that the bacterial viability is not compromised by the presence of the efflux inhibitor itself, each compound was employed at a concentration of 1/2 of the MIC. Exceptions to this were PAN and RES; in the case of PAN, it is known to affect the cellular viability or membrane permeability even at 1/2 of the MIC [13, 30]. In these specific cases (PAN and RES) the compounds were used at a maximum of 20 mg/L (value well below their pre-determined MIC). A reduction in the MIC of at least 1/4 of their original values in the presence of the efflux inhibitor was considered indicative of the presence of efflux activity. All assays were performed in triplicate.

As can be seen by the data presented in Tables 1 and 2, there were significant reductions in the MIC values in the presence of the efflux inhibitors. For example, in the case of the *E. coli* strains there was a considerable reduction of the MIC of TET in the presence of the three compounds tested. This was observed in the TET-resistant strain and one the clinical isolates that showed lower levels of fluorescence (when compared with the control strain). Other reductions of the MIC were also obtained with CIP in the presence of the three inhibitors. The same was obtained for the *Enterobacter aerogenes* clinical isolates. Examples for these strains are shown in Table 1. While the clinical isolate HMEA11 showed a reduction of the MIC to TET in the presence of CPZ, the MIC for NOR was reduced in the presence of CPZ as well as TZ. For the *Salmonella* strains an example is presented for TET and CIP, showing that TZ contributed to a

Table 2. The Effect of Selected Efflux Inhibitors on the Resistance of Induced and MDR Gram-positive Bacteria

Species	Strains	MIC for NOR (mg/L)			
		-	+ TZ	+ CPZ	+ RES
<i>S. aureus</i> ^a	ATCC25923	0.5	0.125 (4×)	0.125 (4×)	0.25
	ATCC25293 _{EtBr}	2	0.25 (8×)	0.25 (8×)	0.5 (4×)
	HEMSA 3	256	128	64 (4×)	128
	HEMSA 4	512	128 (4×)	128 (4×)	256
	HEMSA 5	64	16 (4×)	16 (4×)	32
	HEMSA 9	128	32 (4×)	64	64
		MIC for TET (mg/L)			
<i>Enterococcus</i> ^a		-	+ TZ	+ CPZ	+ RES
	EFC ATCC29212	4	4	4	4
	HSEFM-D	16	4 (4×)	4 (4×)	8
	HSEFM-E	0.125	0.125	0.125	0.125

TET - tetracycline; NOR - norfloxacin; CIP - ciprofloxacin; TZ - thioridazine; CPZ - chlorpromazine; RES - reserpine. ^aparcial data previously published in [21]. EFCATCC - *Enterococcus faecalis* reference strain; HSEFC - *Enterococcus faecalis*; HSEFM - *Enterococcus faecium*. Solutions of TZ and CPZ (Sigma Aldrich, SA, Madrid, Spain) were prepared in deionized water; RES (Sigma Aldrich, SA, Madrid, Spain) was prepared in dimethylsulfoxide. Aliquots of stock solutions of the phenothiazines CPZ and TZ were protected from light and stored at -20°C. Working solutions were prepared on the day of the experiment. To assure that the efflux inhibitors do not compromise the bacterial viability, each compound was employed at a concentration of 1/2 of the MIC. An exception to this is RES, known to affect the cellular viability even at 1/2 of the MIC [30] and in this case the compound was used at a maximum of 20 mg/L (value well below their pre-determined MIC). Values in bold-type correspond to a decrease of 4-fold or higher on the MIC values in comparison to the ones in the absence of inhibitor. Values in parenthesis indicate the MIC decrease relative to the one of the original culture. A reduction in the MIC of at least one-fourth of their original values in the presence of the efflux inhibitor was considered indicative of the presence of efflux activity. All assays were performed in triplicate.

reduction of the MIC (4-fold reduction) of these antibiotics (Table 1). In the case of the *Acinetobacter* strains showed a

reduction of the MIC of MER in the presence of CPZ and PAN. In the case of the Gram-positive strains, *Staphylococ*

Table 3. Chemical Structures of EtBr, Antibiotics and Efflux Inhibitors Used in this Method

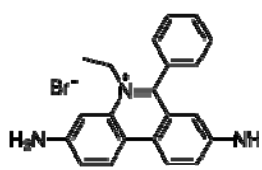
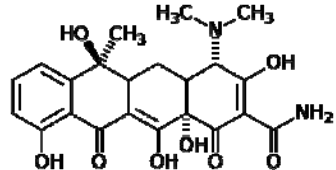
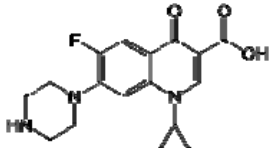
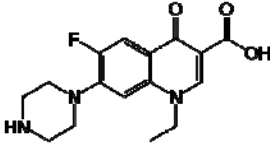
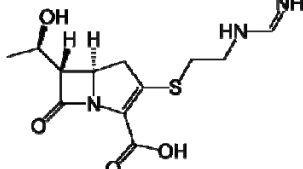
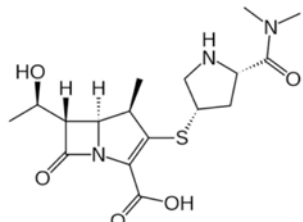
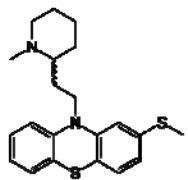
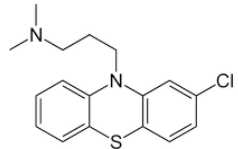
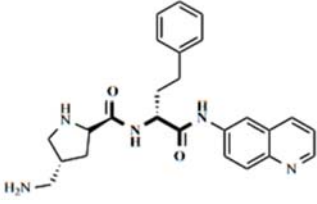
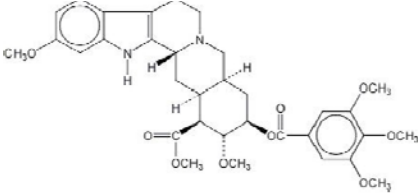
Compounds	Designation	Acronym	Chemical Structure
DYE	Ethidium Bromide	EtBr	
ANTIBIOTICS	Tetracycline	TET	
	Ciprofloxacin	CIP	
	Norfloxacin	NOR	
	Imipenem	IMI	
	Meropenem	MERO	
EFFLUX INHIBITORS	Thioridazine	TZ	
	Chlorpromazine	CPZ	

Table 3. Contd.....

Compounds	Designation	Acronym	Chemical Structure
	Phenylalanine-Arginine beta-Naphthylamide	PAN	
	Reserpine	RES	

cus and *Enterococcus* there was a reduction of the MIC for NOR in the presence of TZ and CPZ for almost all of the strains tested; this reduction varied from 4- to 8-fold. Reserpine was able to reduce the MIC of NOR in the case of the EtBr-induced strain. In general, it was possible to confirm various degrees of efflux activity in the strains tested and that were previously identified and selected by the EtBr-agar cartwheel method.

CONCLUDING REMARKS

Bacterial resistance to antibiotics has become a serious concern for the public health setting. Concomitantly, the role that efflux systems play in antibiotic resistance in MDR bacteria is an important subject that has been extensively discussed in recent years [1, 3, 22, 28]. Although high-level resistance may not occur as a result of MDR efflux pumps alone, the association of over-expression of specific genes among highly resistant clinical isolates cannot be ignored [3, 24-26]. Therefore, we should bear in mind that the intrinsic resistance to antibiotics of some isolates may be largely due to efflux systems [19, 24, 31]. Synergic increases in resistance seen with over-expression of efflux systems, as well as target site mutations can lead to highly resistant bacteria that are difficult to treat with the antibiotics that are currently available. The contribution of efflux to the resistance seen in some clinical strains needs to be considered as one of the parameter used in the design of future antibiotics or any other active compounds [32, 33]. In any event, alterations of the structure of a given antibiotic should be made to reduce the ability of that same antibiotic to be extruded from the bacterial cell, but without compromising its activity [8, 32, 34]. Another approach could be based on potentiating the activity of antibiotics by these inhibitors/novel compounds [19, 20, 30, 32, 33]. The development of compounds that can act as inhibitors/blockers of efflux thereby reducing the impact of these pumps on the efficacy of certain antibiotics, will be of clinical interest and could impact greatly on the clinical setting. However, the major challenge persists: to discover a compound that can be used as an efflux blocker in a non-specific manner, *i.e.*, that can target a big range of efflux systems from different bacterial species. This could be considered a tangible goal as many of these systems show a

high degree of structural homology between different bacterial species [28, 29, 31, 34]. This approach can contribute to the design of more realistic synergic assays that can combine antibiotics and blockers to reduce the MIC for a given antibiotic to a clinical relevant level [20, 32, 33]. That way, bacteria initially resistant to antibiotics can become susceptible. If this approach proves to be successful in the future it can be used as an important alternative in the treatment of some of the most important MDR infections that we have to deal with nowadays [19, 20, 32, 35].

In summary, the EtBr-agar cartwheel method is easy to perform, less time-consuming and can be used to screen large numbers of bacterial strains, thereby facilitating the rapid identification of isolates displaying an MDR phenotype. It can be used in both Gram-negative and Gram-positive clinical isolates to detect MDR mediated by efflux. It is also possible to establish a rapid comparison of efflux activity of laboratory derived isogenic mutants that were manipulated, in the case of strains that were adapted to a given antibiotic by sequential passage exposure; by deletion or interruption of a specific gene or cluster of genes; or by growing the strain in different conditions, for example: different temperature; different pH of the medium; others. Taken together, these factors may play a role in the efflux activity of the isolates, and all of these can be rapidly screened by the described Ethidium Bromide-agar Cartwheel method.

LIST OF ABBREVIATIONS

Bisbenzimidazole	= Hoechst-33342
CIP	= Ciprofloxacin
CLSI	= Clinical and Laboratory Standards Institute
CPZ	= Chlorpromazine
EtBr	= Ethidium bromide
IMI	= Imipenem
MDR	= Multi-drug resistant
MER	= Meropenem

MIC	= Minimum inhibitory concentration
NOR	= Norfloxacin
PAN	= Phenylalanine-Arginine beta-Naphthylamide
RES	= Reserpine
TET	= Tetracycline
TSA	= Trypticase Soy Agar
TZ	= Thioridazine
U.V.	= Ultraviolet

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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