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An Instrument-free Method for the Demonstration of Efflux Pump Activity of Bacteria

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Abstract. *The aim of the study was to develop a simple, inexpensive, reproducible ethidium bromide (EB)-agar based method that is independent of any specialized instrumentation, for the demonstration of efflux pump activity, which is responsible for antibiotic resistance of bacteria. Materials and Methods: A series of agar plates containing varying concentrations of EB were swabbed with strains of Escherichia coli or Staphylococcus aureus, which differed with respect to efflux pump activity. The plates were incubated at different temperatures and time periods and the measurements of fluorescence were used to evaluate the efflux activity of each culture. Results: This simple assay allowed us to identify the efflux of EB in different bacteria following an overnight incubation. The minimal concentration of EB that produced fluorescence was significantly greater at 37˚C than at 4˚C, suggesting the presence of an energy-dependent pump. The method was shown to simultaneously identify strains of a mixed culture that differed from each other with respect to the activity of their efflux pumps. Conclusion: The method, in conjunction with the use of antibiotic-containing disks, provides an additional advantage for the easy identification and selection of colonies that differ with respect to antibiotic susceptibility and degree of efflux pump activity. Because the method is very reproducible it may form the basis for inter-laboratory standardization of efflux pump activity of multi-drug resistant (MDR) clinical isolates.*

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Efflux pumps that are capable of extruding one or more unrelated antibiotics before reaching their targets have been demonstrated in all bacterial species studied to date (1-10). Although there are a number of different classes of such efflux pumps, most of the studies have utilized ethidium bromide (EB) as a substrate (2-4, 6, 7, 9). Consequently, the demonstration and evaluation of efflux pump activity of over twenty-two species of bacteria have involved the extrusion of EB when the pump is active and its retention whenever an agent is present which has the capacity to inhibit the efflux pump (2-4, 6, 7, 9). Other methods, based on radio-labeled antibiotics, have also been employed for the demonstration of efflux pumps which extrude the given antibiotic when active and retain it when their activity has been inhibited (11). However, both the EB and the radio-labeled antibiotic methods are labour intensive, require specialised instrumentation and under the best conditions, yield little precision. Moreover, the means by which the assay is conducted is a marked departure from any physiological situation that is relevant to the activity of the efflux pump. Firstly, any agent that kills the cell or causes an interruption of the cell envelope will cause the build-up of EB within the cell in accordance with the concentration gradient of EB itself. Once EB penetrates the cell it can reach and bind to a large number of internal targets. Hence that amount of bound EB is taken out of the concentration

gradient formula and more will enter the cell. When this takes place, the viability of the cell is reduced or totally obviated given the toxic nature of the compound. If an efflux pump activity is to be demonstrated, the agent used must not affect the viability of the cell under study as is the case when, for example, the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) is used (13).

The essential aspect of all efflux pumps that have the capacity to extrude antibiotics is their dependency on cellular energy derived from the enzymatic hydrolysis of ATP. Whereas simple efflux pumps such as the NorA and Qac systems of *Staphylococcus aureus* and the more complex tripartite efflux pumps of Gram-negative bacteria obtain their energy from the proton motive force (12), it is the hydrolysis of ATP by kinases or ATPases from which the proton gradient necessary for the proton motive force is established. As this enzymatic process is temperature dependent, it should be a simple matter to utilize temperature differences as the means by which one can demonstrate efflux pump activity, provided, that is, one can assess the activity of the pump itself. We have developed a simple method that utilizes the fluorescent efflux pump substrate EB as the means by which one can identify the presence of an efflux pump in a stationary phase bacterial agar culture. The basis for the method lies in the premise that extrusion of the EB is reduced or obviated by a reduced temperature and that this reduction will be manifested in the retention of the fluorescent molecule by either the isolated colony or confluent bacterial mass.

Materials and Methods

Bacteria. The following bacteria were employed in this study: *Escherichia coli* K12 (AG100-main efflux pump acrAB intact), *E. coli* (AG100A-main efflux pump *acrAB* deleted -*ΔacrAB*) and the progeny of the AG100 strain induced to high level resistance to tetracycline (TET) (AG100 *acrAB* TET) were produced in our laboratory and have been described previously (13); *S. aureus* strain ATCC25923 and *S. aureus* methicillin-resistant (MRSA) strains COL (15) and HPV107 (16) were generously provided by Prof. Dr. H. de Lencastre; *Enterobacter aerogenes* EA27 resistant to chloramphenicol has been maintained and characterized by J.M. Pagès (11). Details of culture are described in the appropriate legends of the text.

Materials. Luria Bertani (LB) and Tryptic Soy Broth (TSB) media employed for broth and agar based cultures for Gram-negative bacteria and *S. aureus* strains, respectively, were purchased from Difco (Madrid, Spain). Antibiotics (as powder or as Kirby-Bauer disks) were purchased from Sigma-Aldrich Química SA (Madrid, Spain). EB was purchased from Sigma-Aldrich Química SA. Preparation of agar medium containing varying concentrations of EB is described in the appropriate legends of the text. The effect of temperature on the minimal concentration of EB that results in fluorescence was demonstrated as follows. Two sets of agar plates containing EB concentrations ranging from 0.05 to 0.6 μg/mL were swabbed with *S. aureus* ATCC25923 and incubated overnight at 37˚C. After this period, the results were recorded and one set re-incubated at 37˚C whereas the duplicate set was transferred to 4˚C. At the end of an additional 24-h period, the minimal concentration of EB that produced fluorescence at each temperature was compared to that evident after the first incubation at 37˚C. To determine whether the method could distinguish a mixed culture that consisted of strains of *E. coli* or *Staphylococcus aureus* that differed from each with respect to the degree of efflux pump activity, from each separate culture, 100 μl were transferred to a single tube containing 10 ml of saline, mixed, and from this 10 μl were transferred to another tube containing 10 ml of saline. These two dilutions, each containing a mixture of both strains, were plated onto a replicate series of agar plates containing concentrations of EB from 0.2 to 2.2 μg/ml. Evaluation of fluorescence from the excitation of EB by UV light was first made with the use of a UV transilluminator and the plates photographed with the use of the Eagle Eye (Strategene, USA).

Table I. *The minimum inhibitory concentration (MIC) of EB against bacteria.*

Bacterium	MIC for EB (μg/ml)
E. coli K-12 AG100 (*acrAB*)	150
E. coli K-12 AG100 (*acrAB* TET)	>200
E. coli K-12 AG100A (*ΔacrAB*)	5
Enterobacter aerogenes EA27	>150
S. aureus ATCC25923	5
MRSA COL	10
MRSA HPV107	>30

The MIC for EB was determined by the use of the broth dilution method previously described (13).

Results

The use of a system that is to contain an agent for the demonstration of an efflux pump or its activity must first be preceded by studies that determine the effect of that agent on the growth of the bacteria under study (14). As evident from Table I, EB has differential activity against the bacteria listed. Therefore, the concentrations of EB present in the agar that is to be streaked or swabbed with the bacterial inoculum must be well below those that have an effect on growth. Furthermore, because EB will fluoresce under UV light, the selection of the maximum concentration of EB in the agar must be one that is below the resolution of the UV detector employed. With these considerations in mind, the maximum concentration of EB in agar was below 7.5 μg/ml. Examples of the growth at 37˚C of streaked *S. aureus* ATCC25923 strain in agar containing concentrations of EB that ranged from 0.0 to 4.0 μg/ml demonstrate that at a concentration of 0.4 μg/ml EB bacteria begin to exhibit fluorescence, whereas the agar-EB itself does not exhibit any

detectable fluorescence at a concentration as high as 4.0 µg/ml of EB (Figure 1). The use of swabbing affords certain advantages over the streaking method such as providing a more representative and perhaps uniform result if the strain employed is homogeneous with respect to the presentation of fluorescence at a given concentration of EB. As shown by Figure 2, swabbing of *S. aureus* ATCC25923 strain yields the same result – namely that the minimal concentration of EB that produces significant fluorescence is identical to that yielded by the streaking method (0.4 µg/ml). As the concentration of EB is increased, more fluorescence is associated with the confluent mass. Figure 2 also demonstrates that the minimal concentration of EB that produces fluorescence of the ATCC strain is markedly lower than that of two MRSA strains COL and HPV107, the latter of which has an MDR phenotype.

The effect of temperature on the retention of EB is shown by Figure 3. The transfer of the EB plates that had been incubated at 37˚C to 4˚C resulted in fluorescence of the cultures at a concentration that produced no fluorescence at 37˚C. The set of duplicate plates returned to 37˚C maintained the same minimal concentration of EB that produced fluorescence after the first 24 hours at 37˚C (data not shown).

The minimal concentration of EB that produced fluorescence by different bacteria cultured on EB-containing agar and which have different degrees of efflux pump activity is summarized by Table II. The demonstration of fluorescence associated with the bacterial mass takes place at different concentrations of EB for each of the bacteria studied; the transfer of these plates to 4˚C reduced the minimal concentration of EB that produced fluorescence (Table II). These assays have been conducted on at least six separate occasions, each time in duplicate, and the data obtained has been consistent. Colonies from the 4˚C or 37˚C plates that showed fluorescence at a minimal concentration of EB associated with the cell mass when transferred to the appropriate broth (TSB or LB) were as viable as their respective initial controls (data not shown). Therefore, the presence of EB associated with the bacterial cell mass does not result in any measurable lethality.

To determine whether the method allows the distinction between strains of one species that differ significantly from each other by the activity of efflux pumps, *E. coli* K-12 AG100A (*ΔacrAB*) and the AcrAB over-expressed strain (AG100 TET) were separately cultured in LB broth overnight. Since *E. coli* K-12 AG100A (*ΔacrAB*) and *E. coli* K-12 AG100 (*acrAB* intact) strains are resistant to kanamycin (KAN) and TET, respectively (13), separate disks containing these antibiotics were placed on each EB-agar plate. As shown in Figure 4, the colonies of the KAN-resistant strain, AG100A (*ΔacrAB*), that grow up to the KAN disk exhibit heavy fluorescence, whereas those that grow around the TET disk, the AG100 (*acrAB* intact), strain do not fluoresce. These results show that whereas the strain which has the main efflux pump deleted (*ΔacrAB*), as expected, cannot extrude the EB at concentrations above 0.4 µg/ml, the strain that has the intact acrAB efflux pump readily extrudes EB.

Table II. *Lowest concentration of EB that produced fluorescence associated with bacterial cell mass after 24 hours at 37˚C, after 48 hours at 37˚C and after transfer from 24 hours at 37˚C to 4˚C.*

Bacterium	Lowest EB concentration producing fluorescence (µg/ml)		
	After 24 h at 37˚C	Transfer to: 4˚C	After 48 h at 37˚C
E. coli K-12 AG100 (*acrAB*)	0.4	0.1	0.4
E. coli K-12 AG100 (*acrAB* TET)	2.0	0.1	2.0
E. coli K-12 AG100A (*ΔacrAB*)	0.1	<0.1	0.1
Enterobacter aerogenes EA27	1.9	0.1	1.9
S. aureus ATCC25923	0.4	0.05	0.4
MRSA COL	1.6	0.5	1.6
MRSA HPV107	2.2	0.5	2.2

From overnight cultures in LB or TSB broth, 100 µl were transferred to 10 ml of saline, mixed, and one sterile cotton swab dipped into the mixture, dabbed onto the inside of the tube, and replicate LB or TSB agar plates containing concentrations of EB ranging from 0.0 to 2.2 µg/ml were extensively swabbed to yield a uniform distribution of cells. The plates were incubated for 24 h at 37˚C and then illuminated briefly by a fluorescent lamp and the lowest concentration of EB associated with uniform fluorescence of the colonies recorded. One set of replicate plates were returned to 37˚C and the other replicate transferred to 4˚C. The plates were examined periodically and the lowest concentration of EB that produced uniform fluorescence of the colonies present recorded.

The results presented in Figure 5 show that the colonies of MRSA HPV107 growing right up to the disks containing the OXA, ERY, and CIP begin to show evidence of fluorescence at 1.8 µg/ml of EB, whereas those present in the areas that are far from these disks begin to show fluorescence at 0.4 µg/ml. Removal of single white colonies from the areas around the antibiotic-containing disks and fluorescent colonies from the EB plates containing 0.4 µg/ml of EB with subsequent culture and testing for susceptibility confirmed that the white colonies were of the HPV107 MDR strain and those that fluoresced were the ATCC strain. Transfer of these plates to 4˚C reduced the minimal concentration of EB needed to produce fluorescence of the colonies surrounding the disks (data not shown).

Discussion

The extrusion or retention of the efflux pump substrate EB has been the primary means by which the activity of bacterial efflux pumps has been primarily assessed by most

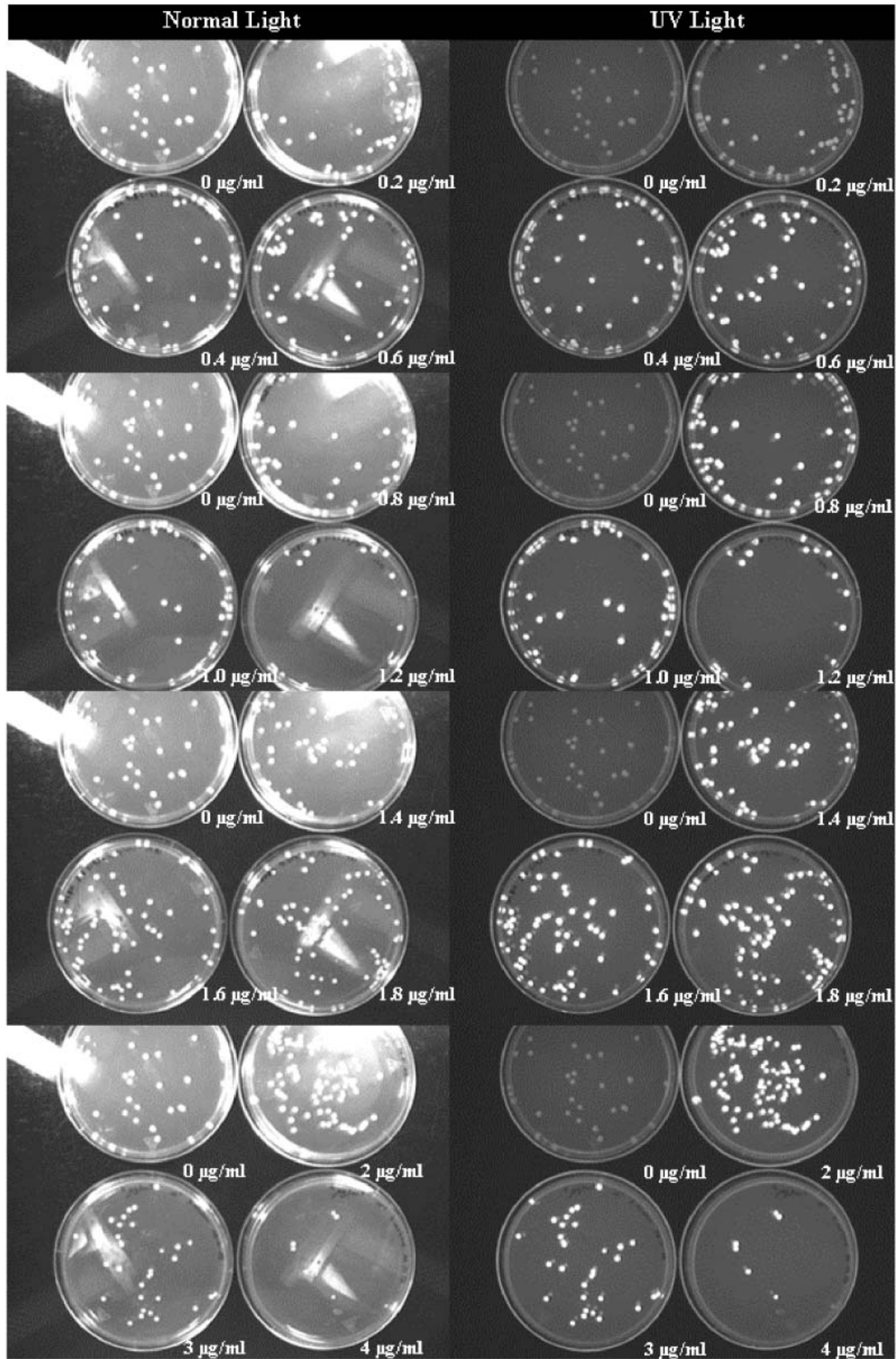

Figure 1. *Fluorescence associated with isolated S. aureus colonies grown on agar containing increasing concentrations of EB.*
S. aureus ATCC25923 grown overnight in TSB was diluted with saline to yield approximately 100,000 cells per ml. This dilution was further diluted 100-fold and from this a small loopful streaked onto TSB agar containing concentrations of EB ranging from 0.0 to 4.0 µg/ml. Plates were incubated overnight and examined under UV illumination. Photographs were obtained with the aid of the Eagle Eye (Stratagene, USA) instrument. Note. Fluorescence of the colonies is associated with the minimal concentration of EB-agar of 0.4 µg/ml. Also note that concentrations of EB as high as 4.0 µg/ml do not produce any fluorescence from the agar itself.

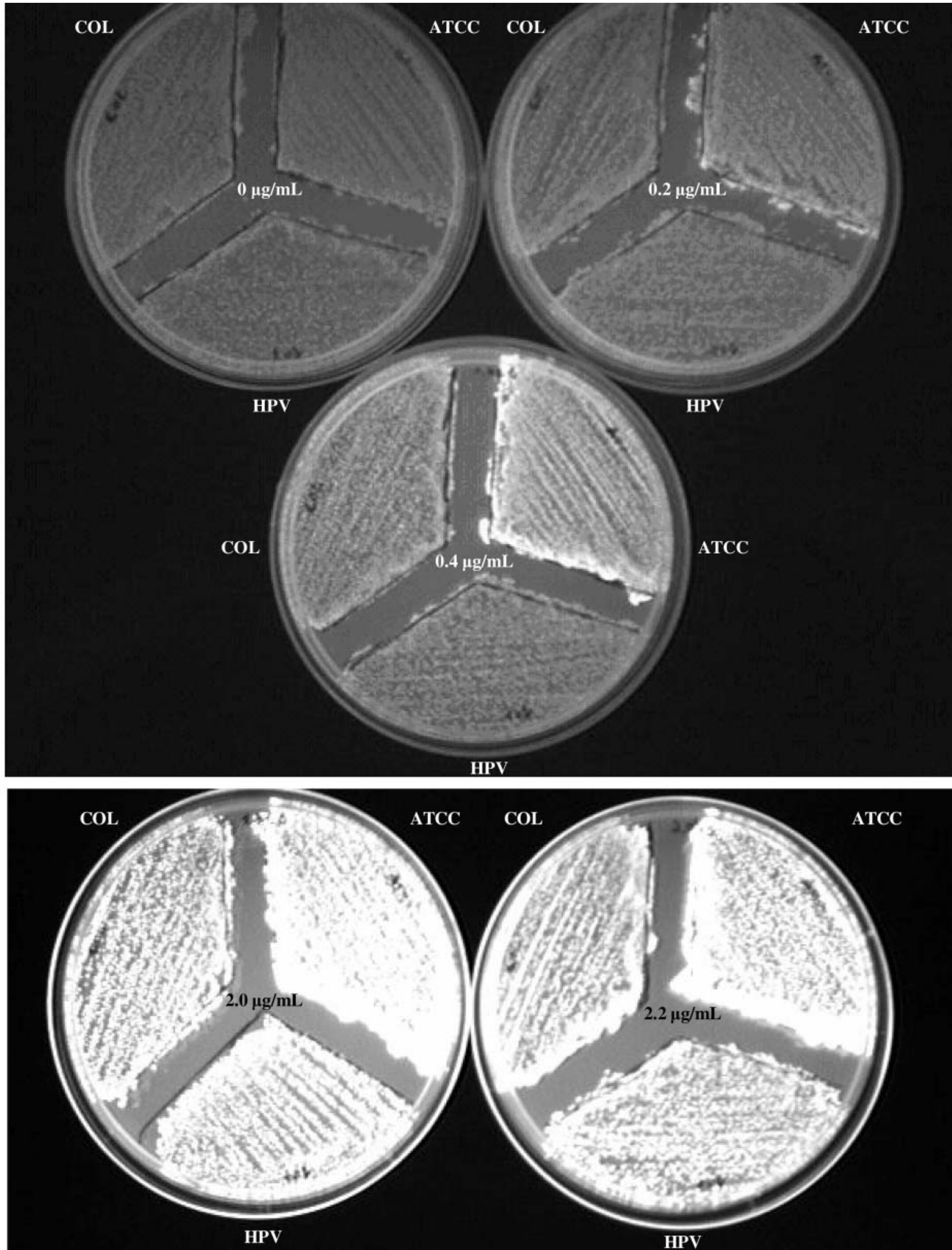

Figure 2. *The minimal concentration of EB that produces fluorescence of S. aureus ATCC, MRSA COL and MRSA HPV107 MDR phenotype. Strains of S. aureus were grown overnight in TSB and 10 μl aliquots were diluted in 10 ml of saline. With the aid of a sterile swab, individuals sectors of each agar plate, which contained various concentrations of EB was swabbed with the ATCC, MRSA COL and MRSA HPV107 strains, respectively. Note. The minimum concentration of EB producing fluorescence of the ATCC strain was 0.4 μg/ml whereas the MRSA COL and HPV107 strains did not fluoresce until a concentration above 1.8 μg/ml of EB was used.*

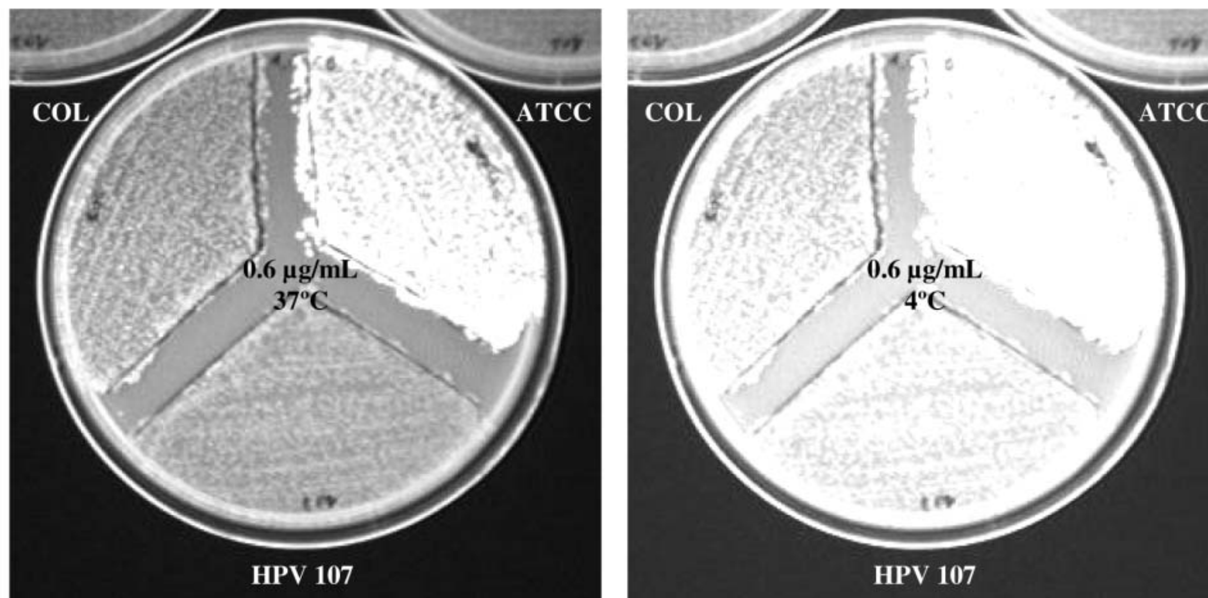

Figure 3. *The effect of temperature on the minimal concentration of EB that produced fluorescence of S. aureus ATCC, MRSA COL and MRSA HPV107. Agar plates containing EB were examined for concentrations that did not produced fluorescence of MRSA COL and HPV107 strains but that did produce ample fluorescence of the ATCC strain. These plates were transferred to 4˚C and examined after 24 h. The minimal concentration of EB that produced fluorescence was reduced from about 1.8 to <0.5 µg/ml (example shown is for 0.6 µg/ml and shows highly evident fluorescence).*

studies. Because efflux pumps are temperature dependent one could predict that the extrusion of EB would be reduced by decreasing the temperature in which a stationary culture is maintained. The results obtained in this study show exactly this – namely, that the fluorescence manifested by this agent, associated with a colony or confluent bacterial mass maintained at 37˚C, is evident at a given concentration of EB. The simple transfer to 4˚C will markedly reduce the concentration of EB that produces similar fluorescence. The manifestation of fluorescence at a lower concentration of EB produced at 4˚C must be the result of retention of this compound as opposed to an increase in the diffusion into the bacterial cell, since the rate of diffusion at 4˚C would be much lower than that taking place at 37˚C. The viability of cells cultured on agar containing concentrations of EB associated with the onset of fluorescence has been evaluated with the aid of colony forming units (CFU) when colonies showing fluorescence at minimal concentrations of EB were transferred to saline, diluted and aliquots then cultured in TSB or LB, depending on the species of bacteria under study, and evaluated at intervals by the use of the CFU method, they were found to have growth rates indistinguishable from those of controls pre-grown in EB-free broth (data not shown).

The results presented in this study demonstrate that the degree of efflux pump activity between strains of bacteria, as well as species, can easily be assessed by the use of the

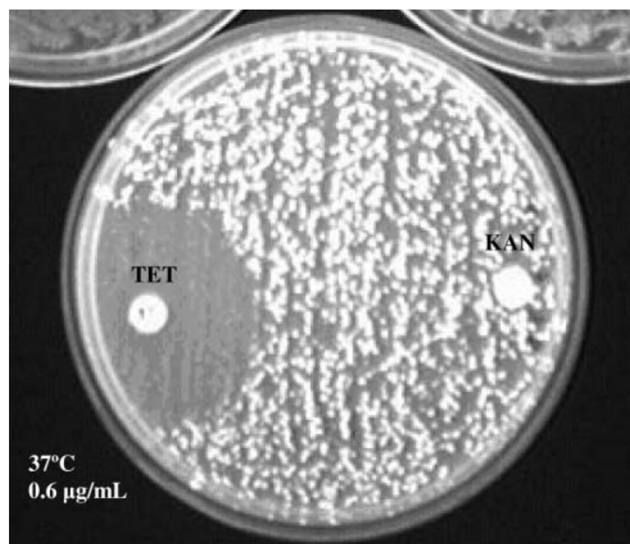

Figure 4. *The use of the EB-agar method for the demonstration of different strains of E. coli that differ with respect to the absence and over-expressed AcrAB efflux pump.*
The EB-agar plate was swabbed with a mixture of acrAB deleted E. coli AG100A, resistant to KAN, and acrAB E. coli AG100, which has high level resistance to TET (13). The plate was incubated at 37˚C, examined and photographed. Note: Colonies around the TET disk that do not fluoresce are those that contain an over-expressed intact acrAB operon which is the cause of high level resistance to this antibiotic whereas those that surround the KAN disk have the acrAB operon deleted and these fluoresce at a concentration of 0.6 µg/ml of EB.

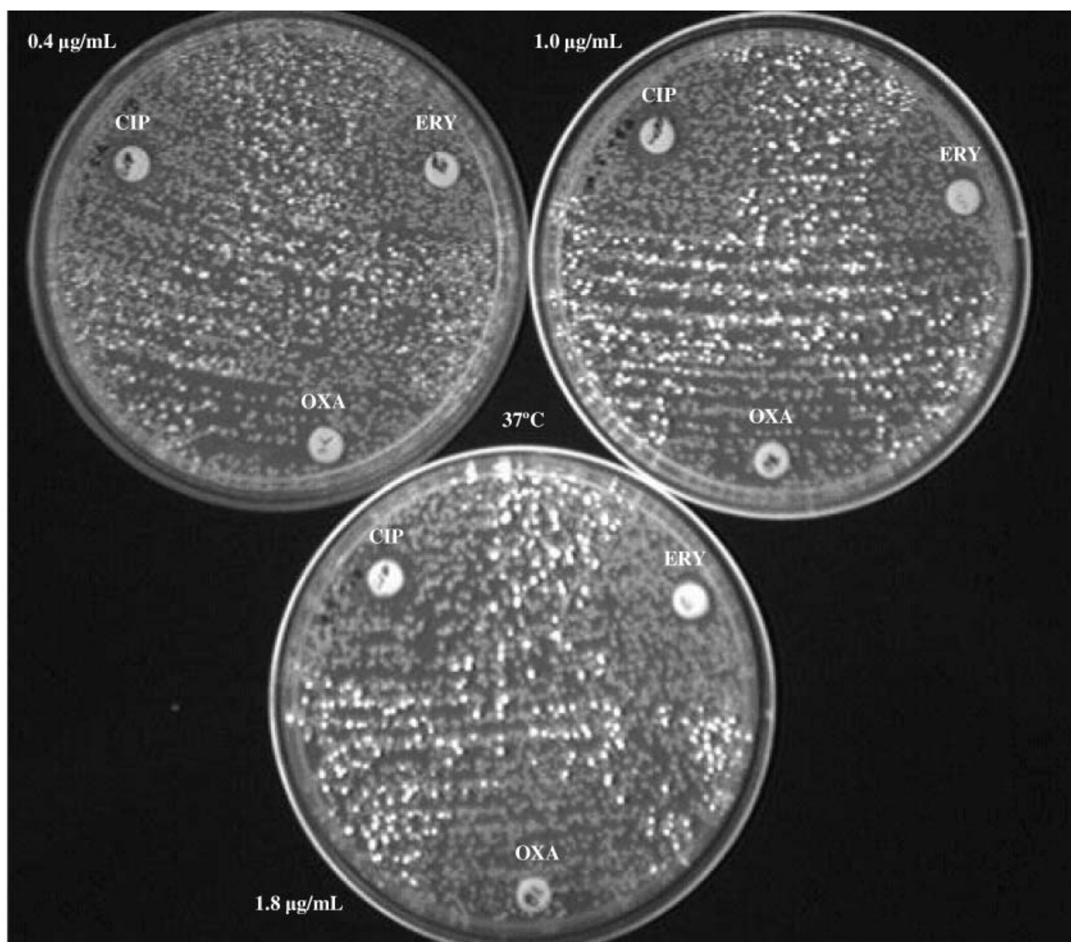

Figure 5. *The ability of the method to distinguish the antibiotic-susceptible S. aureus ATCC25923 strain from the antibiotic-resistant MRSA HPV107 strain. Aliquots of 10 µl of S. aureus ATCC25923 and 40 µl of MRSA HPV107 strains from overnight cultures were added to 10 ml of saline and from this 10 µl were diluted 100-fold with saline. The mixture was swabbed onto EB-containing plates and antibiotic susceptibility disks containing CIP, ERY and OXA applied, and the plates incubated overnight at 37˚C. Note: Colonies growing right up to the disks of CIP begin to show evidence of fluorescence at 1.8 µg/ml. The zone of inhibition noted is devoid of colonies that fluoresce. Isolation of non-fluorescent colonies and fluorescent colonies were later shown by susceptibility assay to be MRSA HPV107 and S. aureus ATCC strains, respectively.*

EB-agar method requiring no specialized instrumentation. The method affords a level of precision that may, after extensive study, provide the means by which efflux pump activity can be evaluated by a consortium of clinical laboratories. If the method progresses to that point, it may find its way into the clinical laboratory and provide the clinician with an additional laboratory tool that identifies the source of MDR of a given bacterial clinical isolate.

The method may prove extremely useful when introducing plasmids that contain individual genes that code for distinct components of efflux pumps into bacteria. It would be expected that when these genes are expressed, the colonies on the agar surface would exhibit fluorescence at significantly higher concentrations of EB than those colonies not having the plasmid-carrying gene (uninfected colonies). If the resolution of this discrimination is sufficiently high, then one may expect that the method may allow the use of plasmids that do not contain antibiotic-resistant genes, required by other methods for the selection of plasmid-carrying efflux pump colonies. The ability to use plasmids that are free from antibiotic resistant marker genes means that larger genes may be introduced without having to increase the size of the plasmid. Moreover, the smaller the plasmid used, the greater its retention by the bacterium, hence, the higher the efficiency of recovery.

The advantages provided by the method transcend its simplicity. Firstly, an agar culture containing a mixture of colonies that differ with respect to the degree of efflux pumps expressed can be replicate plated to agars containing increasing amounts of EB. This affords easy identification,

quantification and isolation of the phenotypes exhibited. Secondly, the replicate cultures themselves can then be transferred to agar containing a more limited range of EB concentrations in combination with concentrations of an agent that is being examined for the inhibition or activation of efflux pump(s). The applications of this method can be further extended to the rapid characterization of bacteria harboring plasmids containing genes that code for distinct components of specific efflux pumps, their regulators and sensors. Variations of the replicate plating can also be tested, in order to evaluate the possible automation of the method, adapting it to microwell-plate technology.

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