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The inhibitory effect of fluphenazine decanoate and caffeine on *Staphylococcus aureus* efflux pumps

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ABSTRACT

The role of Staphylococcal efflux pump in antibiotics and biocides resistance considered global issue and finding cheap, easy to handling and nontoxic efflux pump inhibitors is a persistent need since inhibition of efflux pumps would increase the susceptibility of pathogenic bacteria and restore the antibiotics/biocides activity and considered as critical criteria depended in studying extruding ability in efflux proteins. In this study, the inhibitory effect of two inhibitors (fluphenazine decanoate and caffeine) was investigated in 94 isolates of *S.aureus* isolates selected from 183 isolates according to resistant MDR pattern, 36 of them were positive for efflux activity detected using cartwheel method and confirmed by estimation MIC level of benzilkonium chloride, cetrimide, chloroxylenol, and chlorohexidine gluconate, the obtained results of this study showed that fluphenazine decanoate and caffeine, in the presence of low concentration of ethidium bromide as indicator for efflux activity, are potential inhibitors as the Staphylococcal EP activity to extrude of ethidium bromide was inhibited completely at 1mg/l of fluphenazine decanoate and 100mg/l of caffeine.

Keywords: *Staphylococcus aureus*, Efflux Pumps, Efflux pumps inhibitor, Ethidium Bromide.

1. INTRODUCTION

Quaternary ammonium compounds and biguanides are mainly used in hygiene administration of hospitals, laboratories, houses, skin decontamination and cleaning of surgical equipment (1). Excessive and unappropriated consumption of such compounds interferes with the existence of some pathogenic microorganisms related to multi drug resistant hospital acquired infection since such compounds cause selective pressure leading to antibiotics cross-resistance (2,3). Main mechanism which reduced bacterial susceptibility to biocides is protein network impeded in plasma membrane called MDR efflux pumps, MDR efflux pump transfer vast range of substrates included biocides, metal ion, antibiotics by process known as active efflux, although efflux pump is occupied by bacterial cell and not act as true resistant mechanism but their overexpression provide survival advantages (4,5) besides their actual physiological role as signal transporter for virulence activation, secretion of adherence factors and toxin (6,7,8). Efflux protein belong to five family of transporter included ABC cassette superfamily which depend on ATP hydrolysis as energy source, the multidrug and toxic-compound extrusion family (MATE), resistance nodulation division superfamily (RND), the small multi drug resistance superfamily (SMR) and The major facilitator super family (MFS) (9,10). *Staphylococcus aureus* is intrinsic pathogen and frequent cause of human skin, wound and deep tissue infection, pneumonia, septic arthritis, septicemia and nosocomial infection in addition to food poisoning, since first isolation in 1884 (11,12), these days, less than 90% of *S.aureus* strains resistant to penicillin derivatives, tetracycline, fluoroquinolones, aminoglycosides, macrolides and chloramphenicol which emerge occupied as part of methicillin resistant *Staphylococcus aureus* resistant profile (Jang, 2016) the MDR character in MRSA isolates contributed by array of chromosomal and plasmid efflux pumps that confer resistant to antibiotics and biocides (13,14). Dyes such as ethidium

bromide (EtBr), rhodamine 6G, acriflavine and pyronin Y applied in many research procedures including DNA staining and tracking the transport processes in both microbial and eukaryotic cell (15). In particular, EtBr known as good efflux substrate that used for efflux activity detection synergistically with efflux pump inhibitor (16). The current study evaluate the resistant profile of 183 isolates of *Staphylococcus aureus* isolated from different clinical sources at Baghdad hospitals and investigated the efflux activity among resistant isolates using EtBr agar based method, MIC and efflux inhibition by caffeine and fluphenazine decanoate.

2. MATERIALS AND METHODS

2.1 Collection and characterization

Two hundred isolates of *Staphylococcus spp*. obtained from different clinical sources include urinary tract infection, wound, foot ulcer of diabetic patients, bactremia, burn, ear, oral and nasal infection, these isolates collected from different hospitals in Baghdad city include: Al-Karama hospital, AL -Wassety hospital, Al-Yarmouk hospital, AL-Kindy hospital, Ibn AL-baldi hospital, Al-Emam Ali hospital and Al-Sadr hospital. All isolates transferred with transferable swaps and cultured on brain heart agar plates, after incubation for 18h at 37°C, all isolates cultured on mannitol salt agar and prepared for routine biochemical tests included catalase, oxidase and coagulase considering Bergey's manual of systematic bacteriology (William *et al*., 2009). Only one hundred and eighty-three isolates were able to ferment mannitol, negative to oxidase and positive to catalase and coagulase.

2.2 Genomic DNA extraction

In order to confirm the biochemical diagnosis, the bacterial genomic DNA extracted using salting out method (17) to use as template for *nuc* gene detection which considers critical features distinguish *S.aureus* from other Staphylococcal species. All bacterial isolates activated through transferring single colony to 10ml of BHB containing 2ml/mg of ampicillin and incubated overnight at 37°C, all solution prepared as recommended by Kieser (33). After incubation period the biomass separated by centrifuge at 8000rpm for 15min, the pellet resuspended in 1ml of STE buffer in addition to 100µl of lysozyme solution (30mg/ml) and incubated at 37°C for 1h. After incubation period, 240µl of freshly prepared 10%SDS added to tubes then incubated at 55°C for 90min followed by addition of 800µl of 5M NaCl solution, 10min, 5ml of chloroform was added to tube and homogenized by vortex then centrifuged. The aqueous layer transformed and genomic DNA precipitated by cold isopropanol then centrifuged again. The DNA pellet resuspended with elution buffer (tris-EDTA) later and stored at freezing condition.

2.3 Amplification of *Staphylococcus aureus* diagnostic gene (*nuc*)

Amplification mixture was prepared as follows: (1X) of GoTaq®Green Master Mix provided by (promega/USA), which consist of Taq DNA polymerase, deoxynucleotides (dNTP), $MgCl_2$, reaction buffer, and two dyes (green and yellow) as progress indicator during electrophoresis, the concentration of *nuc*-F and *nuc*-R primers was (10pmol), 50ng of DNA template and free-nuclease water was added to accomplish a total volume 25µl, primer sequence of nuc-F was (GCGATTGATGGTGATACGGTT) and nuc-R (AGCCAAGCCTTGACGAACTAAAGC) (18).

2.4 Susceptibility profiling assay

The susceptibility of 183 isolates toward cefoxitin (FOX30), ceftriaxone (CRO30), meropenem (MEM10), norfloxacin (NOR10), ciprofloxacin (CIP5), levofloxacin (LEV5), trimethoprim (TM5), erythromycin (E15), tetracycline (T10), tigicycline (15) and mecillinam (10) accomplished depending on the instruction recommended by CLSI (19), the inoculum prepared through culturing several pure colonies in 5ml of brain heart broth and incubated at 37°C for 4-6h, the turbidity of broth compared to 0.5 Macfarland standard then swapped on Muller Hinton agar plates, incubated and inhibition zone read depending on CLSI, (19).

2.5 Evaluation of efflux activity by Cartwheel method

Cartwheel method (21) used to evaluate presence or absence of efflux activity within ninety four selected isolates according to multi drug resistant pattern, all selected isolates activated on 5ml of trypton soy broth (TSB) containing 2mg/ml of ampicillin and incubated at 37°C for 18h, the inoculum diluted with normal saline solution to become equal to optical density 0.6 at 600nm then 10µl of diluted inoculums streaked on serious of EtBr-Trypton soy agar (0, 0.25mg/l, 0.5mg/l, 1mg/l, 2mg/l and 4mg/l), the EtBr-agar plates prepared instantaneously and EtBr added to agar before solidifying at 45-50°C. After incubation for 18h at 37°C the result conducted using gel documentation system to detect the absence of EtBr fluorescence in bacterial masses that cultured on EtBr-TSA plates which consider as indicator for efflux activity, negative isolates for efflux pump developed with florescent at low concentration of EtBr (0.25mg/l), all results compared to negative control plates. The result confirmed using the micro dilution method to estimate the minimum inhibitory concentration for some widely used biocides included cetrimide (stock solution 10mg/ml of DMSO), benzylkonium chloride 50%, chloroxylenol (stock solution 10mg/ml of DMSO) and chlorohexidine gluconate 4%.This study used the protocol recommended by CLSI, (22,23).

2.6 Efflux detection synergistically efflux pumps inhibitor and EtBr

The EtBr agar method used in combination with fluphenazine decanoate, 2,4dinitrophenol, verapamil and caffeine at low concentration of (0.25 mg/l) EtBr, plates of EtBr containing efflux pumps inhibitors prepared at the same day of experiments and florescence detected after overnight incubation of the cultured plates by gel documentation through comparison with control plate.

3. RESULTS AND DISCUSSION

According to the result of biochemical (oxidase, catalase, coagulase) and molecular (*nuc* gene) routine diagnostic procedures, one hundred and eighty three isolates were confirmed as *Staphylococcus aureus* among 200 collected Staphylococcal samples (91.5%) from different clinical sources included foot ulcer, urine, wound, burn, ear, nasal, oral and blood from different hospitals in Baghdad city. The sensitivity result revealed that the great majority of isolates were MDR isolates most of isolates were resistant against more than two antimicrobial agents (24). The cefoxitin discs were used to differentiated between MRSA and MSSA isolates depending upon the diameter generated by antibiotics discs (19), the result were found out that 66.12% were MRSA isolate developed without inhibition zone of cefoxitin, all isolates that resistant to cefoxitin were also resistant to ceftriaxone and meropenem (66.12%). The resistant percentage toward fluoroquinolones group (norfloxacin, ciprofloxacin, levofloxacin) was 28.41%. 76.50%, 51.91%, 49.72%, 23.49% and zero was the resistance percentage to mecillinam, tetracycline, erythromycin, trimethoprim and tigicycline respectively. The sensitivity profile of MRSA isolates that were resistant to cefoxitin displayed in figure (1).

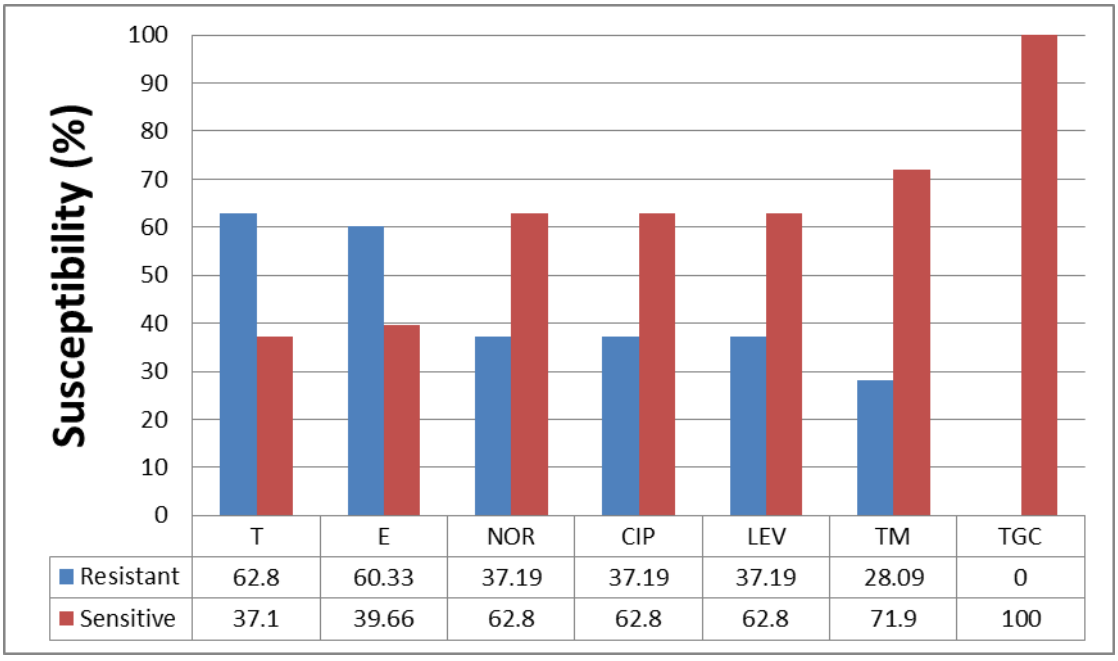

Figure 1: Resistance percentage of MRSA isolates to antibiotics, antibiotic abbreviations are: T (Tetracycline), E (Erythromycin), NOR (Norfloxacin), CIP (Ciprofloxacin) LEV (Levofloxacin), TM (Trimethoprim), TGC (Tigecycline).

The results of sensitivity assay of this study were approximately similar to the local studies at different years included 2017 (25), 2015 (26), 2013 (27) and 2011 (28) in Baghdad city. Most of the applied antimicrobial agents considered target for Staphylococcal efflux pumps (2), among 183 isolates, 94 isolate selected according the MDR resistant patterns, especially the isolates that were resistant to (fluoroquinolones group) because such antibiotics considered as good efflux pumps target. The detection of efflux pumps handled using concentration series of TSA-EtBr agar plates, each plate with specific concentration cultured with 8 isolates in the form of cartwheel, after incubation period the result documented under UV light using gel documentation device detect the presence or absence of fluorescence in the mass of bacterial growth, presence of flourscnce within bacterila cell at low concentration mean negative result because the bacterial cell don't have efflux pumps that enable it to extrude EtBr in opposite to positive isolates as represented in figure (2).

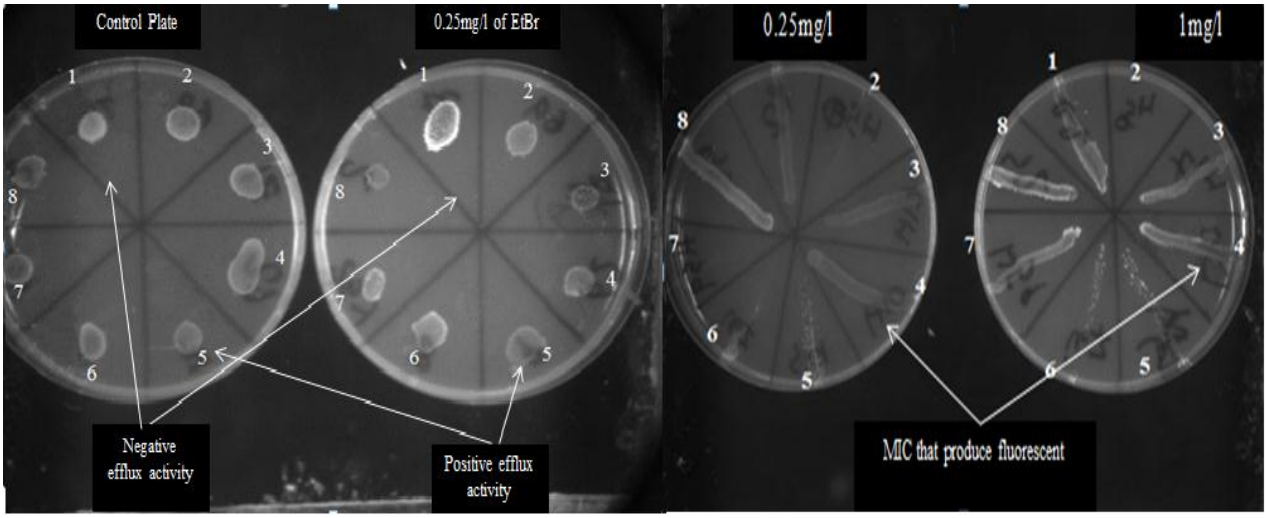

Figure 2: Presence and absence of fluorescence associated with S.aureus efflux pumps on 0.25mg/ml of EtBr-TSA plates, All isolates on section (2-7) documented as positive, isolates in section 1 represented negative isolate.

Interestingly, the result detected thirteen isolates with higher efflux activity (at concentration 2mg/ml) in comparison to the activity level of other isolates, finally, sex isolate only showed no fluorescent at 4mg/ml of EtBr). The isolates that showed no fluorescence on 2mg/ml or higher documented as isolates in overexpression situation for efflux pumps determinants, these result either from enhancing the expression of efflux pumps since EtBr consider substrate of many Staphylococcal efflux pumps so that the substrate will effect on regulator gene, or these isolates belong to special MRSA clone characterized by overexpression of efflux determinants.The result of all EtBr assay at different concentration listed in table 1.

Table 1: All selected 94 isolates of S.aureus subjected to different concentration of EtBr.

Situation	0.25mg/l	0.5mg/l	1mg/l	m2mg/l	4mg/l	8mg/l	
Positive	46	36	17	13	6	Out resolution	
Negative	45	49	57	59	60	Out resolution	
No growth	3	6	11	2	6	23	Total (51)

The total number of *S.aureus* isolates that killed by EtBr was 51 isolates distributed at different concentration (0.25-8mg/l) which consider an evident indicates that EtBr have variable activity against bacteria (20). As result of exposure to EtBr, one of the isolate transform to mutant at concentration 4mg/l which consider high concentration, the mutant isolates produce flourscence at concentration 0.25-2mg/l but the fluorescent absent completely at 4mg/l as showed in figure (3).

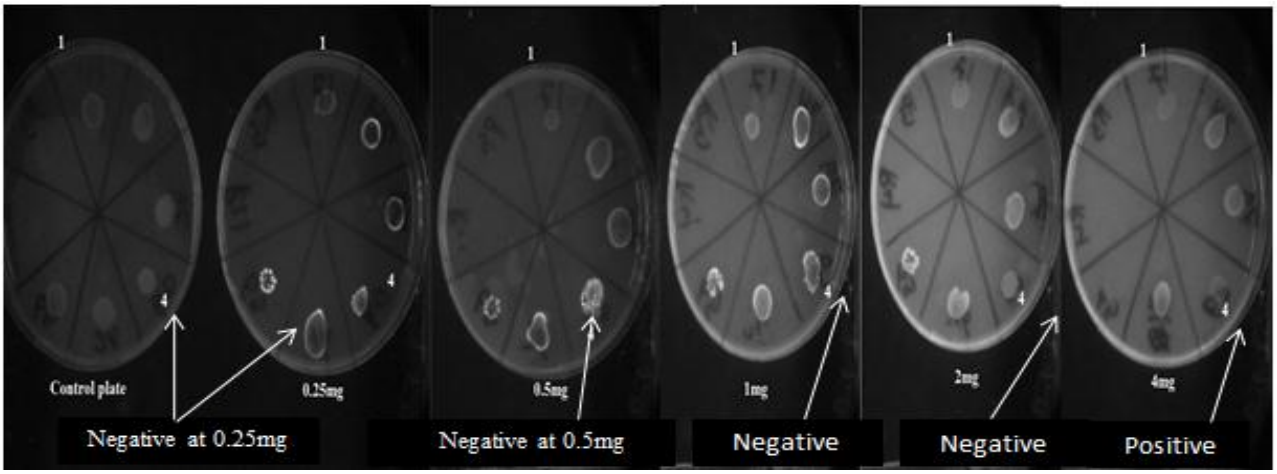

Figure 3: Mutant isolate production using EtBr as result of high concentration, the mutant isolates was negative at all plates from 0.25mg/ml to 2mg/ml. at 4mg/ml the isolates transform into positive.

The transformation in phenotypic expression situation can result from mutation at promoter region or at regulatory genes of efflux pumps genes leading to overexpression proteins after subjecting to sublethal dose of antiseptic/dye compounds (5). Number of 8 isolates which characterized by highly overexpression level of efflux pump selected to confirmed the result of cartwheel assay through determining the minimum inhibitory concentration (MIC) level for some widely used antiseptics included chloroxylenol, benzylkonium chloride, cetrimide, and chlorohexidine gluconoate. All selected isolates showed highly resistant level to all used antiseptics which used at concentration ranged from commercially used concentration to much higher level. The MIC for all 8 *S.aureus* isolates toward PCMX (2.44 -1250 µg/ml) was higher than 1250 µg/ml, the result showed that PCMX had no effect on these bacterial isolates when compared with negative and positive control, however this result expected depending on World health organization (WHO); cloroxylenol (PCMX) less effective against *Staphylococci* bacteria (30), these isolates also exhibit high resistance level >1250 µg/ml to cetrimide (2.44- 1250 µg/ml) and > 500 µg/ml for BK, and were resistance to the commercially dose of CHX that applied in mouth wash antiseptic solution 2%.

All 94 isolates cultured on TSA plates contained 1mg/l of fluphenazine decanoate and 0.25mg/l of EtBr, all isolates compared with the same isolated cultured on other plates containing 0.25mg/l of EtBr only. The fluphenazine decanoate record efflux inhibition for all positive isolates at 0.25mg/l of EtBr, the efflux inhibition also recorded using caffeine at concentration 100mg/l of caffeine as represented in figure (4). The result also compared with isolates negative to efflux inhibition, the effect of verapamil and 2,4 dinitro phenol at different concentration also examined on efflux positive and negative isolates but no inhibition recorded although applying wide range of concentration. This study provides the first report that showed the role fluphenazine decanoate as efflux pumps inhibitor through application with cartwheel assay instead of using expensive chemical compounds as inhibitors that widely used in efflux pumps detection research such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) and phenylalanine-arginine-β-naphthylamide (PAβN) (29). Inhibition mechanisms is not clearly defined but it supposed that the inhibitor bind directly to the pump or with the substrate, inhibitors can lead to energy depletion by disrupting proton gradient or prevent ATP- binding besides that inhibitor can form large complex with substrate so that not extruded by efflux activity, as a result of inhibition the bacterial cell will lose ability to form biofilm because of the correlation between efflux pumps and biofilm formation since transporting system play critical role in signal transport (cell to cell) of biomolecules that participate in biofilm formation (32,31).

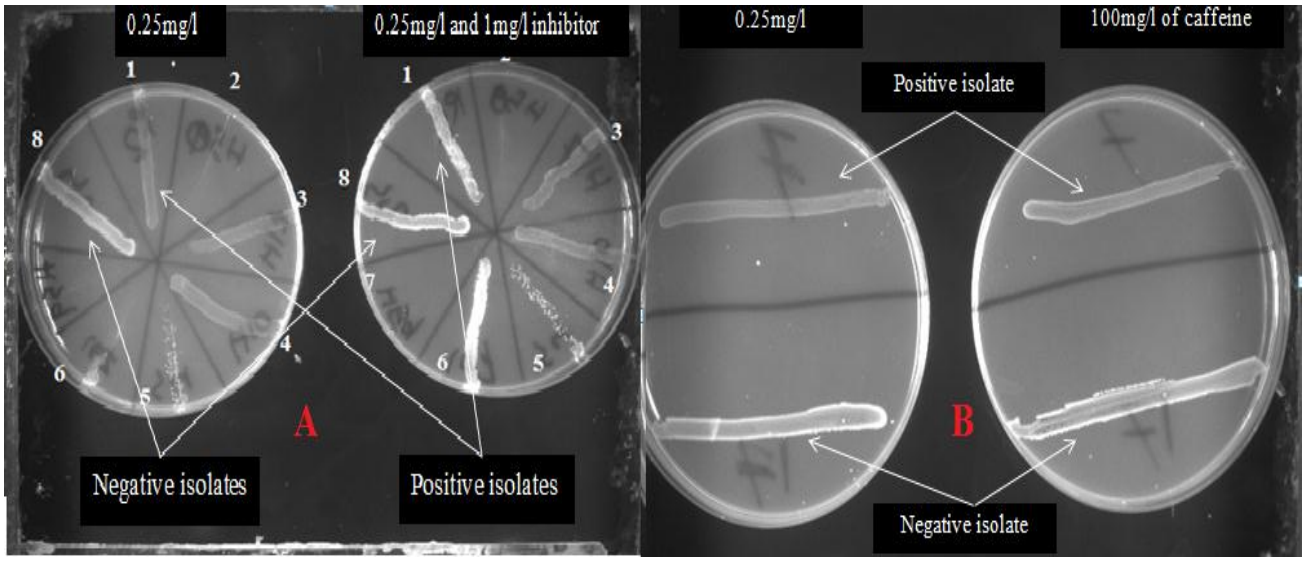

Figure 4: Inhibition of efflux transporter using cheap substrate, A- fluphenazine decanoate, B- Caffeine. The effect of inhibitor on positive and negative isolates detected at 0.25mg/l of EtBr and by comparing the result with other plates contains EtBr only.

4. CONCLUSION

The study reports the emergence of efflux activity in multi-drug resistant Iraqi hospital isolates of *S. aureus* and the role of efflux pumps in antibiotics and biocides resistant phenotypes as result of cross-resistance between dyes/biocides and antibiotics resistant. The obtained result showed that isolates with higher efflux activity were more resistance to biocides comparing with low efflux activity isolates that were more susceptible to biocides. Therefore, implying detection methods like ethidium bromide agar cartwheel for efflux pumps activity in routine lab work can be used for rapid detection of antibiotic/biocide multi-drug resistant bacteria instead of familiar MIC measurements. Fluphenazine decanoate (1mg/l) and caffeine (100mg/l) are good candidates as a cheap inhibition of efflux pumps activity.

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