MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF EFFLUX PUMPS AND EFFLUX PUMPS INHIBITOR ON ANTIBIOTIC RESISTANCE OF *SALMONELLA TYPHI* IN BAGHDAD

Mustafa Faris Husain **Nuha Joseph Kandala**
Researcher **Prof.**
Dept. of Biotechnology, Coll. of Sci. University of Baghdad, Iraq.
Mostafa.Fares1206a@sc.uobaghdad.edu.iq **nuhanajeeb@sc.uobaghdad.edu.iq**

ABSTRACT

The aim of the study was investigate the occurrence of multidrug resistance efflux pumps genes in local clinical isolates of *Salmonella sp* and try to apply efflux inhibitor for phenotypic detection besides their possibly future therapeutic uses to retain the activity of some frequently used antibiotics. Among 114 bacterial specimens obtained from several hospitals in Baghdad city only 67 isolates were diagnosed to *Salmonella typhi* according to conventional and molecular methods. The disk diffusion (Kirby Bauer) method was adopted to assess the antibiotic susceptibility of *S. typhi* isolates, and the results revealed variable rates of resistance to various antibiotics. While Gentamicin are the most effective antibiotic in this study since 100% sensitive to it .Cartwheel method used for evaluating the efflux pumps activity by using ethidium bromide in different concentration, the outcomes appeared positive reaction for 42 isolates (62.6%) at 0.25 µg/ml, 25(37.3%) at 0.5 µg/ml , 15(22.3%) at 1 µg/ml , 9(13.4%) at 1.5 µg/ml , 4 (5.9%) at 2 µg/ml, 4(5.9%) at 4µg/ml. Four isolates which characterized highly expression level of efflux pumps activity and high resistant to antibiotic were selected to confirmed the result of cartwheel assay through determining the minimum inhibitory concentration (MIC) level though applied different concentration (500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9, and 1.9) µg/ml of fluphenazine decanoate as efflux pump inhibitor (EPIs) and appeared at 15.62 µg/ml of EPIs the positive activity efflux pumps became negative and Etbr appeared fluorescent.

Keywords: enterobacteriace, fluphenazine decanoate, ethidium bromide, *flic-d* gene.

الخصائص الجزيئية و المظهرية لمضخات التدفق ومثبطات مضخات التدفق على مقاومة المضادات الحيوية للسالمونيلا التيفية في مدينة بغداد

مصطفى فارس حسين **نهى جوزيف قندلا**
باحث **أستاذ**
قسم التقنيات االحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

المستخلص

الهدف من الدراسة هو التحري من وجود مورثات مضخات التدفق المقاومة للأدوية المتعددة في العزلات السريرية المحلية للسالمونيلا و استخدام مثبطات التدفق للكشف عن النمط المظهري إلى جانب استخداماتها العلاجية المستقبلية للاحتفاظ بنشاط بعض المضادات الحيوية المستخدمة بشكل متكرر. من بين 114 عينة بكتيرية تم جمعها من مستشفيات مختلفة في مدينة بغداد تم تشخيص 67 عزلة فقط للسالمونيلا التيفية وفقا للطرق التقليدية والجزيئية. درست حساسية المضادات الحيوية لعزلات السالمونيلا التيفيه باستخدام طريقة انتشار القرص (Kirby Bauer) وأظهرت النتائج التي تم الحصول عليها تغاير في نسب المقاومة للمضادات الحيوية المختلفة. في حين كان المضاد الحيوي الجنتمايسين الأكثر فعالية في هذه الدراسة بنسبة حساسية 100٪. أظهرت نتائج طريقة Cartwheel المستخدمة لتقييم نشاط مضخات التدفق باستخدام بروميد الإيثيديوم بتراكيز مختلفة، تفاعلا إيجابيا ل 42 عزلة (62.6٪) عند 0.25 ميكروغرام / مل، 25 (37.3٪) عند 0.5 ميكروغرام / مل، 15 (22.3٪) عند 1 ميكروغرام/ مل، 9 (13.4٪) عند 1.5 ميكروغرام / مل، 4 (5.9٪) عند 2 ميكروغرام / مل، 4 (5.9٪) عند 4 ميكروغرام / مل. تم اختيار أربع عزلات تميزت بدرجة عالية فوق مستوى التعبير لنشاط مضخات التدفق ومقاومة عالية للمضادات الحيوية لتأكيد نتيجة مقايسة Cartwheel من خلال تحديد الحد الأدنى لمستوى التركيز المثبط (MIC) على الرغم من أستخدام تراكيز مختلف (500، 250، 125، 62.5، 31.25، 15.62، 7.8، 3.9، و 1.9) ميكروغرام/ مل من fluphenazine decanoate كمثبط لمضخة التدفق (EPIs) وأظهرت النتائج عند 15.62 ميكروغرام/ مل من EPIs ، كانت مضخات التدفق الإيجابي للنشاط سلبية وظهر Etbr مشع.

الكلمات مفتاحيه: العائلة المعوية, ديكانوات فلوفينازين, بروميد الإيثيديوم, جين *flic-d*

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INTRODUCTION

Salmonella is considered important public health and economic problems in human and animal's worldwide (18, 24). One of the human-restricted endemic pathogens named "enteric or typhoid fever" is *S. enterica serovar Typhi (S.Typhi)*. In underdeveloped nations, it has been reported that the enteric fever spreads via the fecal-oral route through contaminated food and water (11).The effectiveness of antibiotic therapy is still threatened by antimicrobial resistance, a worldwide public health issue. Bacterial pathogens have evolved into multidrug resistant forms in various forms that have been isolated internationally. There are a variety of processes by which antibacterial resistance develops, including enzymatic inactivation, drug modification, drug target protection, and efflux of medicines through efflux pumps. Both typhoidal and non-typhoidal serovars have been linked to antibiotic resistance (8).In efflux, a molecule is normally transported out of the cell. Efflux pumps play a critical function in the physiology of bacteria by making it easier for chemicals, metabolic waste, and essential nutrients to enter and exit the cell. Pathogenic bacteria efflux systems are typically divided into five categories the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (26).Only gram negative bacteria possess RND pumps, which are structured as a tripartite system with a protein channel on the outer membrane, a periplasmic adaptor protein, and a membrane pump that is attached to the cytoplasm. The fact that all super families of efflux pumps rely on the energy provided by the proton motive force (PMF) is one feature in common; however, only pumps in the ABC superfamily use ATP energy (33).AcrB and its tripartite complex acrAB-tolC, which has a variety of substrates and is the most well-studied RND pump in *Salmonella*, are important mediators of multidrug resistance in Gram negative bacteria, including numerous Enterobacteriaceae (25).Many research techniques, such as DNA labeling and monitoring transport pathways in both microbial and eukaryotic cells, use dyes such ethidium bromide (EtBr), rhodamine 6G, acriflavine, and pyronin Y (10).EtBr is a good efflux substrate renowned for being used in conjunction with an efflux pump inhibitor to detect efflux activity (22).The aim of study assessed the antimicrobial susceptibility of 67 isolates of *Salmonella enterica Typhi* obtained from blood and stool samples in Baghdad hospitals and looked into the efflux pump activity among isolates using the cartwheel method (phenotypic method) and Molecular method by using polymerase chain reaction technique.

MATERIALS AND METHOD

Collection and characterization: One hundred fourteen clinical sample included , Blood specimens eighty one , stool specimens thirty three were collected from patients suffering of enteric fever after gained approval the college of science research ethics committee according to (CSEC\1021\0086) from different local hospitals in Baghdad Baghdad Medical City (National Center for Educational Laboratories, Baghdad Teaching Hospital), Al karama Teaching Hospital , Al-Yarmouk Teaching Hospital for seven months , in dated from January 2022 to July 2022. Each specimen was cultured on specific media MacConkey, Xylose Lysine Deoxycholate (XLD) and *Salmonella Shigella* (SS) agar (Himedia-India). Gram staining identified morphological characteristics and biochemical identification included sugar fermentation, IMVC test (indole, methyl red (MR) Voges proskauer (VP) and Citrate), Oxidase ,Catalase , Motility and Triple Sugar Iron (TSI) (16).

Antibiotics Susceptibility assay

The susceptibility of isolates toward different groups of antibiotic included meropenem (MRP 10), tigecycline (TGC15), levofloxacin (LE5), chloramphenicol (C30), piperacillin (PI 100), polymyxin-B (PB300), cefepime (CPM30), amikacin (AK30), aztreonam (AT30), piperacillin/tazobactum (PIT100), ceftriaxone (CTR30), gentamicin (GEN10), ciprofloxacin (CIP5), ceftazidime(CAZ30), amoxyclav (AMC30) and colistin (CL10) Depending on the guidance provided by CLSI 2022, achieved (5). Antibiotic susceptibility

testing of all isolates was determined using the Kirby-Bauer disc agar diffusion technique, The inoculum was prepared by cultivating a number of pure colonies in 5ml of brain heart broth. (Himedia, India) for 18 hours at 37°C. Before shifting the stock to the Muller Hinton agar plates, the turbidity of the broth was compared to a 0.5 McFarland standard. (Himedia, India), incubated, and the inhibition zone was based largely on Clinical & Laboratory Standards Institute (5).

Evaluation the efflux pumps activity by Cartwheel method: Cartwheel method was used for phenotypic detection of the efflux pump in *Salmonella spp* isolates (19). Briefly, all samples activated on 5ml of brain heart broth (Himedia /India) and incubated at 37 °C for 18 h, In order to achieve an optical density of 0.6 at 600 nm, the inoculum was diluted with 10µl of normal saline solution. The diluted inoculums were then streaked over a plate of EtBr-nutrient agar (NA) (Himedia/India) (0, 0.25, 0.5, 1, 2 and 4) µg/mL , The plates for the EtBr-agar were made immediately, EtBr was poured to the agar before it solidified at 45–50°C . For light protection, the plates were coated in aluminum foil. After an 18-hour incubation period at 37°C, the results were acquired using a gel documentation system to show that bacterial colonies cultured on EtBr-NA plates did not glow with EtBr., which is regarded as a sign of active efflux pumps.

DNA extraction: The DNA was isolated from bacterial growth according to the protocol of Bacteria Genomic DNA kit GBB 100/101-100 preparation Kit (Geneaid, Taiwan). The Quantus Fluorometer (Promega, USA) was utilized to determine the concentration of extracted DNA in order to determine the quality of samples for subsequent applications.

Amplification of *Salmonella enterica Typhi* diagnostic gene (*flic-d*): Amplification mixture was produced as follows: Taq DNA polymerase, deoxynucleotides (dNTP), MgCl2, reaction buffer, and green dye that serves as a progress indicator during electrophoresis are all included in the 10µl ready-to-use New England Biolabs Quick-load® Taq 2x Master Mix PCR. Concentrated amount of *flic-d-F* and *flic-d-R* primers was (10pmol) taken 1µl from each one , 50ng of DNA template used 2µl and free-nuclease water was added to accomplish a total volume 20µl , primer sequence of *flic-d*-F was (5`-ACTCAGGCTTCCCGTAACGC-3`) and *flic-d*-R (5`-GGCTAGTATTGTCCTTATCGG-3`) that will yield 763bp fragment (29). To examine the primer's optimum annealing temperature, the DNA template was amplified with the same primer pair, (Forward) (Reverse), at annealing temperatures of (50, 52, 54, 56, 58 and 60°C). The PCR reaction was carried out with the following : initial denaturation at 95°C for 5min, second denaturation at 95°C for 30 sec, annealing at 56°C for 30sec, extension at 72°C for 30sec and final extension at 72 °C for 7min.32 cycle of amplification was applied. To analyze the PCR products, 10 µl of PCR mixture was loaded to 1.5 % agarose in the presence of 100bp DNA ladder. After performing gel electrophoresis, the gel was exposed to U.V by using U.V Tran's illuminator.

Amplification of Efflux pumps *acrAB-tolc* genes of *Salmonella typhi:* Aliquot of 2µl from 50ng DNA was used as template for PCR. *acrA* , *acrB* and *tolC* genes presences among the extracted DNA of isolates was determined by polymerase chain reaction. The *acrA* forward primer (5`-TGTCACACTAAAAACGGAAC-3`) acrA reverse primer (5`-GACATAAATAGGGTCCAGCT-3`) that yield 517 bp fragment . While the *acrB* forward primer (5`-ATCGTCAGTTCTCTATCACC-3`) acrB reverse primer (5`-TTGGCTTTCTCTTTGTTCAG-3`) that yield 415bp fragment . Finally the *tolC* forward primer and reverse primers were (5`-CAACATGGGGCAGAATAAAA-3`) (5`-CCGAGCGCATATTTGATATT-3`) that yield 384 bp fragment . All these primers as newly designed primers according to (NCBI). A volume of PCR reaction was 20 µl contains 10 µl Taq Quick-Load 2X master mix (Biolabs / England), 1µl from each one forward and reverse primers for one gene, 50ng of DNA template used 2µl and total volume was achieved by adding free-nuclease water. To examine the primer's optimum annealing temperature, the DNA template was amplified with the same primer pair, (Forward)

(Reverse), at annealing temperatures of (50, 52, 54, 56, 58 and 60°C). The PCR reaction was carried out with the following: initial denaturation at 95°C for 5min, second denaturation at 95°C for 30 sec, annealing at 56°C for 45 sec for *acrA*, *acrB* and *tolC* gene, extension at 72°C for 2 min. 35 cycle of amplification was applied. To analyze the PCR products, 10 µl of PCR mixture was loaded to 1.5 % agarose in the presence of 100bp DNA ladder. After performing gel electrophoresis, the gel was exposed to U.V by using U.V Tran's illuminator.

Efflux detection synergistically efflux pumps inhibitor with EtBr: Fluphenazine decanoate (25mg/ml) and a little amount of EtBr (0.25µg/mL) were employed in the EtBr agar method. Plates of EtBr with various concentrations of efflux pump inhibitor (500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9, and 1.9) mg/ml were apparently made and after cultivated plates were incubated overnight, florescence became recognized via gel documenting and comparison with a control plate (28).

RESULTS AND DISCUSSION

Identification of *Salmonella spp.:* During the present study period, 114 blood and stool specimens from patients suffering of enterica fever were collected. Morphological, biochemical and microscopic analyses have been used to identify *Salmonella* isolates for the first time. Microscopically, *Salmonella* was recognized as a gram-negative bacillus that was peritrichous, flagellated, motile, and did not produce spores. These results are the general phenotypic characteristics of *Salmonella* and similar to (16). Although XLD (Xylose Lysine Deoxycholate) agar, SS (*Salmonella Shigella*), and MacConkey agar were used for the morphological analysis of *Salmonella* isolates, *Salmonella* colonies on MacConkey agar, however, appeared pale yellow (non-lactose ferment), 1-3 mm in diameter, and after 18–24 hours at 37°C, as well as good development of *Salmonella* colonies on XLD agar, emerged gray in color with black core colonies because of its capacity to produce H2S. The biochemical outcomes of the *Salmonella* isolates were revealed by the IMVC test, TSI, catalase, Oxidase, Ureases, and Motility test assays. In the TSI slants test, the slant and bottom turned AKL/ACID red and yellow, indicating non-fermentation of glucose on the slant and acid production with H2S in the bottom. Additional testing on Salmonella isolates produced negative results for oxidase, Indole production, urease generation, and citrate utilization. The VITEK-2 GN ID Cards System was used to identify *Salmonella* isolates using a variety of biochemical assays. The results showed a group of *Salmonella* with cards identifying a variety of high-quality isolates (percentage from 95 to 99%) they are illustrats in Table (1). Just sixty seven (58.77 %) isolates were dignosis as *Salmonella spp.* among one hundred and fourteen, which is Approach to that of AL-Quraishi & AL-Amm (2018) (1) and to Hassan *et al*, (2021) (11).The results of *Salmonella enterica* on culture and biochemical test shows in Figure (1) .

Table 1. The biochemical tests to identify Salmonella spp.

Test	Oxidase	Catalase	urease	Citrate	VP	MR	Motility	TSI agar	H2S	Indole	Lactose fermentation MacConkey agar
Salmonella	-	+	-	-	-	+	+	**Ak/A/+++**		-	-

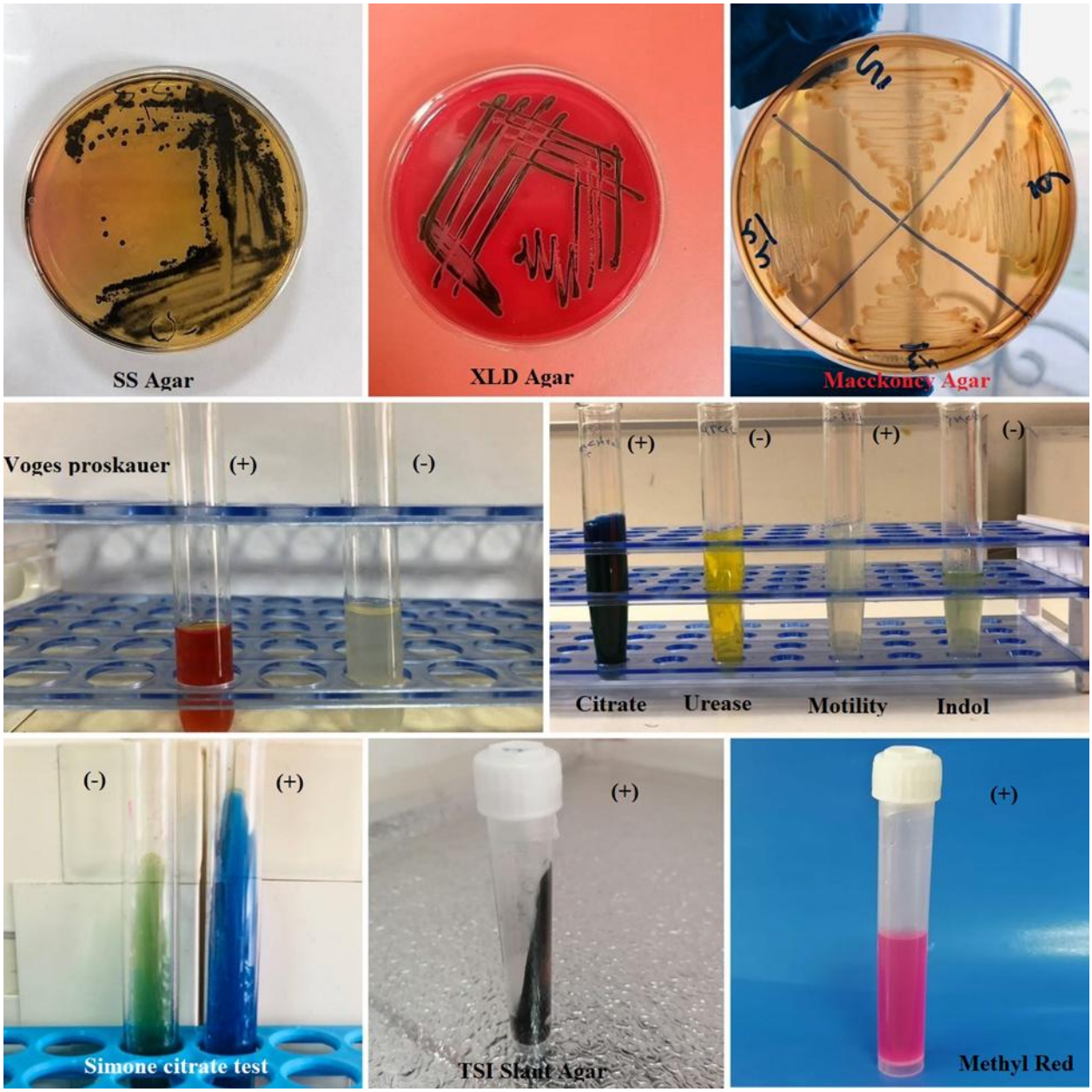


Figure 1. *Salmonella spp.* appear on SS agar which produces H2S will form black center colonies , appear on XLD red colonies with black centers , on mackoncy colorless non lactose ferment and biochemical test (Indol , methyl red, voges proskaure ,Citrate , Urease , motility , TSI).

To confirm the subjected of these isolates , molecular method was using the PCR technique to detect the species of *salmonella .fli-d* gene that identified the *Salmonella* into *serovars typhi* and the results showed that all 67 (100%) isolation was *Salmonella enterica serovar typhi* Figure (2). These results were similar to those of researchers who used the same gene in diagnosing these bacteria to *typhi* and considered this gene to be diagnostic for *Salmonella* typhoid bacteria (23).Khan et al. carried out a study using PCR for the detection of *Salmonella typhi* on culture negative cases of suspected typhoid fever, also compared the results of PCR with blood culture and Widal test and found that PCR was 100% sensitive (12).

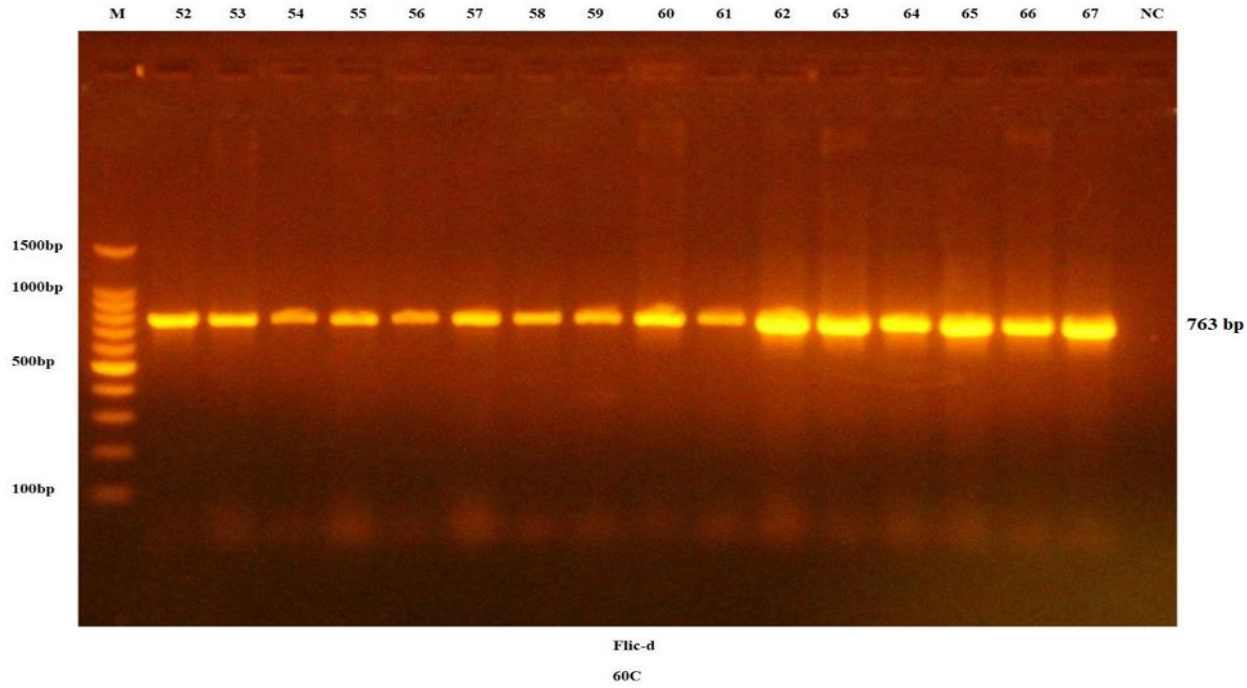
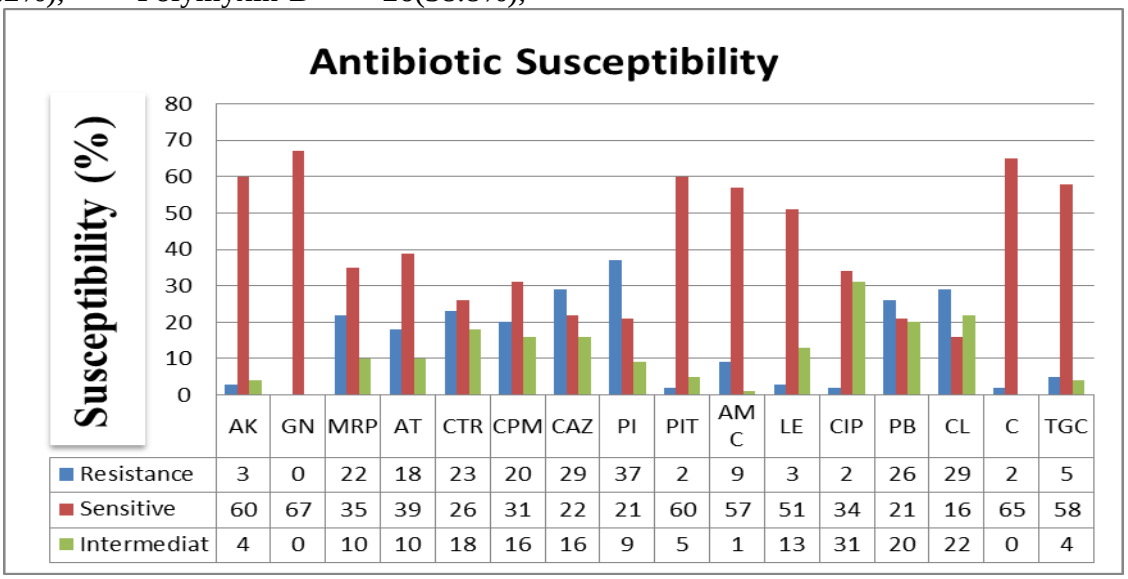


Figure 2. PCR product of *flic-d* gene primer with product size 763 bp gel electrophoresis for 1hr at 7 v/cm by using ethidium bromid dye

Antibiotics Susceptibility assay

The sixty-seven isolates of *S.typhi* bacteria were examined antibiotic disc for its susceptibility using the disc diffusion (Kirby-Bauer) method .The results as show in Figure (3) revealed that *S.typhi* was variant in their resistant to all antibiotics included Piperacillin 37(55.2%), Colistin 29 (43.3%) , Ceftazidime 29(43.2%), Polymyxin-B 26(38.8%), Ceftriaxone 23(34.3%), Meropenem 22(32.8%), Cefepime 20(29.8%), Aztreonam18 (26.8%) , Amoxyclav 9 (13.4%), Tigecycline 5(7.4%) , Levofloxacin and Amikacin each one 3 (4.4%) ,Ciprofloxacin , Piperacillin/Tazobactum and Chloramphenicol each one was 2 (2.9%), while all 67 (100%) isolates were sensitive to Gentamicin.



	AK	GN	MRP	AT	CTR	CPM	CAZ	PI	PIT	AMC	LE	CIP	PB	CL	C	TGC
Resistance	3	0	22	18	23	20	29	37	2	9	3	2	26	29	2	5
Sensitive	60	67	35	39	26	31	22	21	60	57	51	34	21	16	65	58
Intermediat	4	0	10	10	18	16	16	9	5	1	13	31	20	22	0	4

Figure 3. The antibiotic susceptibilitiy test among *Salmonella spp* isolates: Meropenem (MRP), Tigecycline (TGC), Levofloxacin (LE), Chloramphenicol (C), Piperacillin (PI), Polymyxin-B (PB), Cefepime (CPM), Amikacin (AK), Aztreonam (AT), Piperacillin/Tazobactum (PIT), Ceftriaxone (CTR), Gentamicin (GEN), Ciprofloxacin (CIP), Ceftazidime (CAZ), Amoxyclav (AMC) and Colistin (CL).

The finding revealed resistance of isolates to β- lactam antibiotics (penicillic, Cephalosporines, Monobactams, Carbapeneams) this resistance can be conferred by a number of different mechanisms (enzymatic destruction of the

antibiotics, altered antibiotic targets, or decreased uptake of the drug) (7, 35), but the most common and important mechanism is the production of β-lactamases that cleave the amide bond of the characteristic four-membered β-lactam ring, rendering the drug inactive and harmless to bacteria (15,21). The isolates showed resistance to lipopeptide antibiotics (Colistin, Polymyxin-B) this resistance due to reduction in binding capacity with the outer membrane caused by an alteration in lipopolysaccharide (LPS) (14).Gram-negative bacterial outer membrane consists of porins, which are protein channels responsible for transport of nutrients into the cell, Alteration or loss of porins reduces the permeability to antibiotics thereby conferring resistance against β-lactams in organisms such as *P. aeruginosa*, *K. pneumoniae* and *S.enterica*. Other study by Mahmood, (17) for Enterobacteriacea group wich variant in resistant to different antibiotic (Cefepime, Meropenem, Amikacin, Piperacillin, and Aztreonam) in percentages (50%, 40%, 20% ,40% and 55%). Membrane-bound efflux pumps contribute significantly to resistance by driving antibiotics out of the bacterial cells. Some Gram-negative bacteria such as *S.enterica* possess efflux pump mechanisms. The Enterobacteriaceae family has the acrAB-tolC RND system, which has been well characterized (34).

Evaluation the efflux pumps activity by Cartwheel method: One of the ways that bacteria become resistant to antibiotics is through high efflux pump activity. It was found that an organism's capacity to expel EtBr from the cell was a good indicator of the efflux activity of isolates. Various EtBr concentrations were tested using the cartwheel maneuver. All outcomes were compared to negative control plates for the efflux pump negative isolates, which were produced with fluorescent at a low dose of EtBr (0.25 µg/mL). In this study the outcome appeared that 42 isolates (62.6%) at 0.25 µg/mL, 25(37.3%) at 0.5 µg/mL, 15(22.3%) at 1 µg/mL, 9(13.4%) at 1.5 µg/mL, 4 (5.9%) at 2 µg/mL, 4(5.9%) at 4 µg/mL isolates were did not retain EtBr inside the cells indicating pronounced efflux activity (Figure 4). The efflux activities of isolates could be differentiated at EtBr concentrations of 1 µg/mL and 1.5µg/mL. Ethidium bromide (EtBr) is considered as a common substrate of efflux pumps in Enterobacteriaceae (30).The EtBr cartwheel approach has been employed to quickly identify MDR bacteria linked to efflux pumps and basic principle of cartwheel method depend on comparative control for fluorescence analysis not on shapes of strains (20).The intrinsic efflux activity of *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* has been evaluated using EtBr (3,6,27). Antibiotic resistance may not necessarily be linked to efflux pump activity, though. The extrusion of several metabolites, colors, and compounds that are harmful to cells occurs physiologically through the pumps in addition to the development of antibiotic resistance. However, the emergence of multiple drug resistance among bacteria may be significantly influenced by efflux pumps.

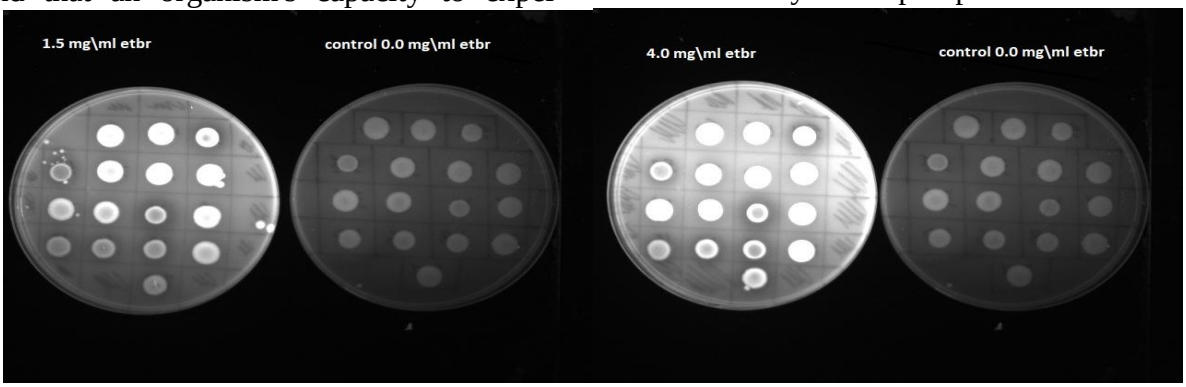


Figure 4. The efflux pumps activity by ethidium bromide (EtBr) cartwheel method on different concentrations of EtBr (0, 0.25, 0.5 , 1 ,1.5 , 2 ,4) µg/mL with nutrient agar. Culture after incubation at 37°C for 24 hr, The plates were visualized in UV illuminator and documented

Amplification of Efflux pumps *acrAB-tolc* genes of *Salmonella typhi:* The detection of efflux pumps by using PCR technique for all sixty seven samples, the results revealed that 52 (77.6%) samples carried *acrA* gene and the PCR product appeard as single DNA band with a molecular base of 517 bp Figure (5). This gene is very important for encoding periplasmic adaptor proteins (PAP) (formerly described as membrane fusion proteins) (32).The outcome of *acrB* gene showed that the percentage of positive result was 48 (71.6 %) samples and the product of PCR which appear as single DNA band with a molecular base of 415 bp Figure (6) . This gene It is the trans-membrane domains of acrB are particularly well conserved with almost all residues being identical, including this amino acid triplet involved in proton translocation suggesting that the mechanism may be common to all RND systems (31). As for the last gene of the efflux pump system, it was the ratio of its presence between isolates of 43 (64.17%) with size of amplification product 384 bp as shows in (Figure 7), The structures of several outer membrane proteins that associate with RND efflux systems have been solved (13).

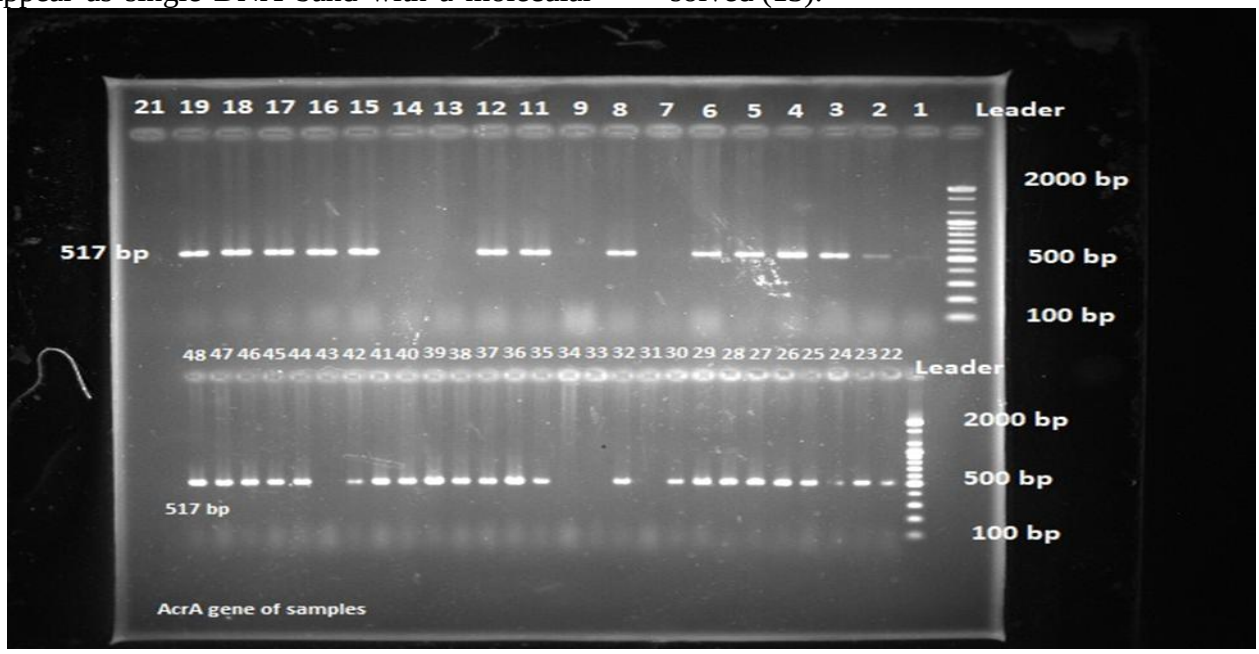


Figure 5. PCR product of *acrA* gene with molecular base of 517 bp in 1.5% agarose gel electrophoresis for 1hr at 7 v/cm by using ethidium bromid dye and visualized under UV light

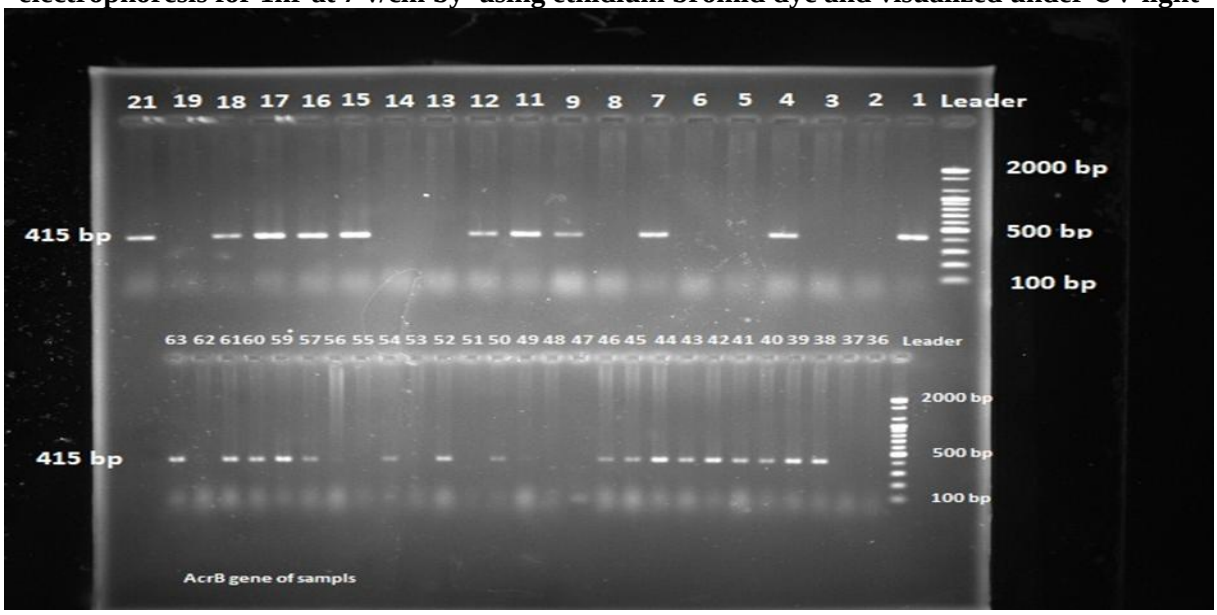


Figure 6. PCR product of *acrB* gene with molecular base 415 bp in 1.5% agarose gel electrophoresis for 1hr at 7 v/cm by using ethidium bromid dye and visualized under UV light

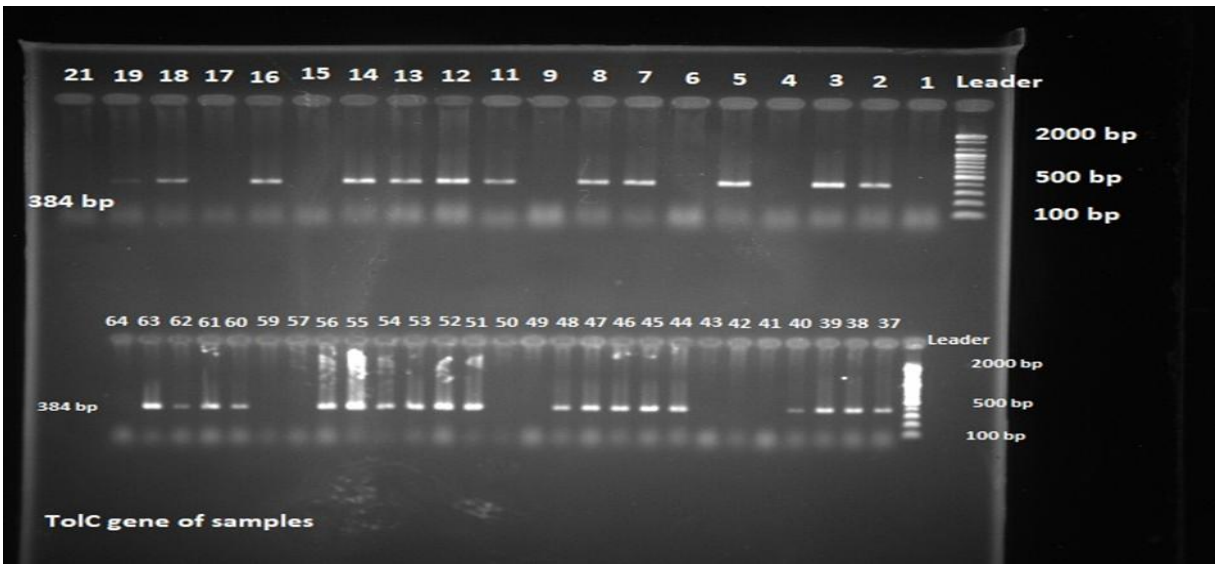


Figure 7. PCR product of *tolC* gene with molecular base 384 bp in 1.5% agarose gel electrophoresis for 1hr at 7 v/cm by using ethidium bromid dye and visualized under UV light

Efflux detection synergistically efflux pumps inhibitor and EtBr: Four isolates which characterized highly expression level of efflux pumps activity and high resistant to antibiotic were chosen to establish the minimum inhibitory concentration (MIC) level in order to corroborate the cartwheel assay results though applied fluphenazine decanoate as efflux pump inhibitor (EPIs). By using different concentration of EPIs (500, 250 , 125 , 62.5 ,31.25 , 15.62 ,7.8 ,3.9 ,1.9)mg/ml and 0.25mg/L of EtBr in NA agar plates . For all positive isolates, the fluphenazine decanoate MIC reveals efflux inhibition at concentrations of 15.62mg/ml and 0.25mg/L of EtBr, the positive activity efflux pumps became negative and retain eithedium bromid inside the cell and became fluorescent (Figure 8).This EPIs used first one by Saber and Kandala, on gram positive bacteria *S.aureus* and achieved success (28). Although the exact mechanism of inhibition is unknown, it is assumed that the inhibitor binds to the pump or substrate directly. In addition, inhibitors can cause energy depletion by interfering with the proton gradient or by preventing ATP from binding Following that, a substantial complex was formed with the substrate, preventing efflux pumps from doing their job of preventing extrusion.

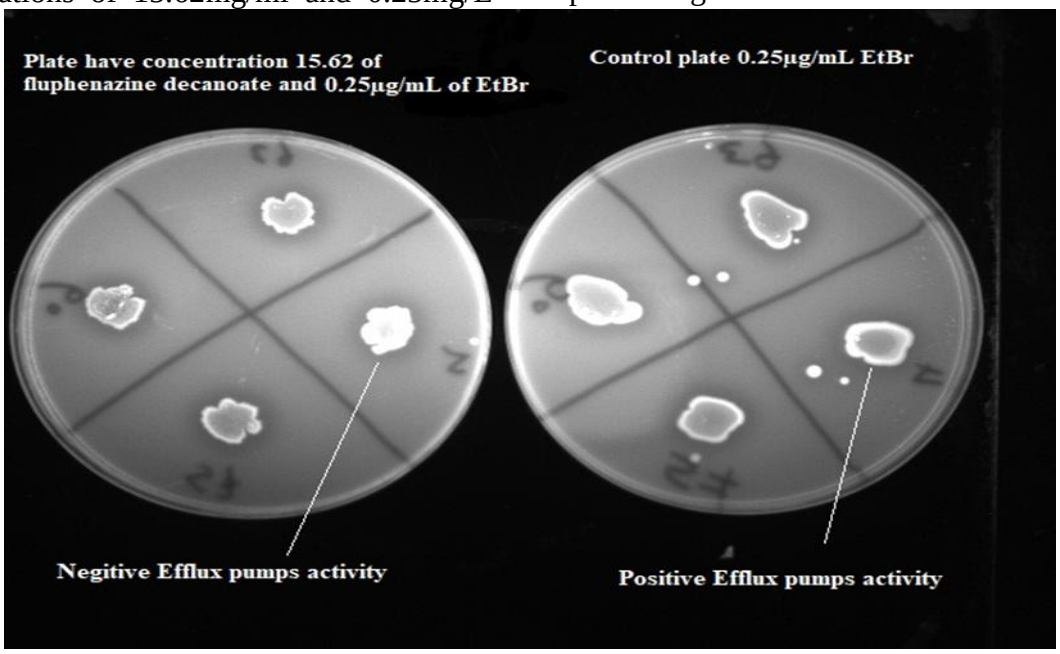


Figure 8. Fluphenazine decanoate's efflux transporter inhibition When compared to other nutrient agar plates that solely contain EtBr, the inhibitor's impact on positive and negative isolates that were found at 0.25 µg/mL of EtBr is shown

CONCLUSION

Lack of efflux activity for EtBr may not necessarily mean that all bacterial strains lack efflux pumps because of the variation in the substrate specificity of the efflux pumps for antibiotics and EtBr. Similarly, it might not be accurate to assume that EtBr and antibiotics will have the same efflux activity. This outcome can suggest efflux pumps inhibitor as promising agent to reduce antibiotics resistant and to enhance its suscptibilility.

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