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Investigations The Bacterial Salmonella Pollution Between Local Slaughtered, Random Slaughter And Imported Chicken Meat In Karbala Province

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Abstract
Salmonella is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria belonging to the family Enterobacteriaceae. These organisms are of critical concern globally due to their significant role in foodborne illnesses and zoonotic diseases. A total of 120 fresh chicken meat samples were collected from five regions in Karbala City, Iraq: Al-Hur, Al-Hindiya, Ain-Tamr, Al-Huseiniyah, and the city center. The samples were categorized into three groups: in each region should be contain 24 samples collection which contain locally slaughtered chickens (8), imported chicken meat (8), and randomly slaughtered chickens (8), form each region of karbala province, From each chicken, thigh and breast cuts were aseptically excised using sterile scalpels and placed in individually labeled sterile bags. Samples were transported under refrigeration (4°C) to the laboratory within 24 hours for further analysis. One gram chicken from each sample for all species with a sterile wood spatula, put it into an Erlenmeyer flask etc. and add 9 ml buffered peptone water , Mix and Incubate at 37°C overnight (18-24 hours) then Transfer 1 ml of the pre-enrichment with a pipette to 10 ml Tetrathionate broth (Müller-Kaufmann). Incubate at 37° C for human sample and 41°C ± 0.5°C for animal samples, overnight (18-24 hours) then Spread a 10 µl loop full from the inoculated and incubated Tetrathionate broth on XLD and on SS agar plates and incubate at 37°C overnight (18-24 hours). Through the current study, it was found that the areas where the infection was recorded were in two regions Al-Hindiya and Al-Husayniya, while the least areas were in the Ain Al-Tamr. The Salmonella species isolated from various aspects of the broiler breeder project environment were distributed among four species, with the following percentages: S. typhimurium: 11 isolates (52%), S. enterditis: 7 isolates (33%), S. goodwood: 2 isolates (10%), S. senftenberg: 1 isolate (5%). bacterium was found on XLD agar with a small red colony and a black center.

Keywords: *Salmonella, PCR, S.Sagar, Efflux Pump*

INTRODUCTION:

Salmonella is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria belonging to the family Enterobacteriaceae (Eng et al., 2015; Ryan et al., 2017). These organisms are of critical concern globally due to their significant role in foodborne illnesses and zoonotic diseases (1). The genus Salmonella comprises two main species, Salmonella enterica and Salmonella bongori, with S. enterica being responsible for the vast majority of human and animal infections (2). With the growing incidence of antimicrobial resistance and the high prevalence of Salmonella-associated diseases in both developed and developing countries, understanding the biology, transmission, epidemiology, and public health implications of this pathogen has become increasingly important (3). The genus was named after the American veterinary pathologist Daniel Elmer Salmon, who first isolated the bacterium in the late 19th century, although it was his colleague Theobald Smith who actually discovered it (Eng et al., 2015). Since then, Salmonella has been extensively studied due to its pathogenic potential, diverse serotypes, and ability to adapt to a wide range of hosts and environments (4). It is estimated that Salmonella causes millions of cases of gastroenteritis and tens of thousands of deaths each year, especially in regions with poor sanitation and inadequate food safety practices (5).

There are over 2600 known serovars (or serotypes) of Salmonella, which are classified based on their O (somatic) and H (flagellar) antigens. Among these, Salmonella enterica serovar Typhi (S. Typhi) and S.

Paratyphi are responsible for causing typhoid and paratyphoid fever, respectively — systemic illnesses that can be fatal without treatment. Other non-typhoidal Salmonella (NTS) serovars such as S. Enteritidis, S. Typhimurium, and S. Heidelberg primarily cause self-limiting gastroenteritis but can lead to severe invasive infections, especially in immunocompromised individuals. The epidemiology of Salmonella infection is complex and multifactorial. Humans can acquire the infection through ingestion of contaminated food or water, direct contact with infected animals, or through person-to-person transmission in certain settings (Eng et al., 2015). Common sources of Salmonella include raw or undercooked poultry, eggs, unpasteurized milk, and contaminated produce (6). In developing countries, poor hygiene, inadequate sewage disposal, and limited access to clean water contribute significantly to the high burden of typhoidal and non-typhoidal salmonellosis (7).

Several studies have reported an increase in antimicrobial resistance in Salmonella strains isolated from foods of animal origin. The spread of this antimicrobial resistance has been largely attributed to the indiscriminate use of antibiotics, both in human and animal therapeutics. Some studies have shown an association between the use of antimicrobials in animals and the development of antimicrobial resistance in bacteria isolated from humans (8).

In the livestock industry, antibiotics are used for therapeutic, prophylactic, or growth-promoting purposes in sub-therapeutic doses for long periods in animals intended for human consumption (Cota E, 2014; Errecalde JO, 2004). This generates antibiotic residues in meat, milk, eggs, and other food products (Márquez LD, 2008), and they have the potential to induce the emergence and selection of resistant bacteria that are transmitted through the food chain (9).

The problem of antibiotic resistance is positioned as one of the key elements for the development of global health, on a par with new pandemics, emerging infections, and global climate change (10).

The study aimed to Evaluate salmonella bacterial contamination and to quantify and compare the levels of bacterial contamination in locally slaughtered versus imported chicken meat as well as to identify the specific bacterial species present in chicken meat

Materials and Methods

Collection of samples

A total of 120 fresh chicken meat samples were collected from five regions in Karbala City, Iraq: Al-Hur, Al-Hindiya, Ain-Tamr, Al-Huseiniyah, and the city center. The samples were categorized into three groups: in each region should be contain 24 samples collection which contain locally slaughtered chickens (8), imported chicken meat (8), and randomly slaughtered chickens (8), form each region of karbala province, From each chicken, thigh and breast cuts were aseptically excised using sterile scalpels and placed in individually labeled sterile bags. Samples were transported under refrigeration (4°C) to the laboratory within 24 hours for further analysis. Take out 1 g chicken from each sample for all species with a sterile wood spatula, put it into an Erlenmeyer flask etc. and add 9 ml buffered peptone water , Mix and Incubate at 37°C overnight (18-24 hours) then Transfer 1 ml of the pre-enrichment with a pipette to 10 ml Tetrathionate broth (Müller-Kaufmann). Incubate at 37° C for human sample and 41°C ± 0.5°C for animal samples, overnight (18-24 hours) then Spread a 10 µl loop full from the inoculated and incubated Tetrathionate broth on XLD and on SS agar plates and incubate at 37°C overnight (18-24 hours) and read the XLD plates and SS plates .Salmonella suspect colonies on XLD and SS agar onto non-selective media, (nutrient agar) plates for biochemical confirmation of Salmonella (11).The bacteria isolated from human and animal sources were taken from an overnight culture of transport media and streaked on Macconkey agar and nutrient agar, after that, isolates suspected to be of interest were subculture onto Brilliance Salmonella agar to ensure pure growth

VITEK®2 for Confirmatory identification:

VITEK-2 system (Biomerieux, France) with a planned identification card was working in this study for confirmation. The 64-well card contains 43 colorimetric substrates for phenotypic identification species of bacterial, such as S. Typhimurium after purified onto selective medialike XLD and incubated at 37°C for 24

hr. then, a suspension of the bacteria was prepared in saline (0.45% to 0.50% NaCl) put in a polystyrene tube to a density equivalent to a McFarland tube number 0.5. The density was determined using Vitek2 DensiChek spectrophotometer then, the tube and card were inserted into the Vitek2 cassette, and the card was auto-inoculated within the Vitek 2 instrument by a method depended on vacuum-release. Wells in the card are easy notice scanned and read each 15 minute, with a total number of incubation time which about 8 hours (12).

DNA Extraction and molecular investigation:

Genomic DNA was isolated from bacterial growth according to the protocol of Addbio DNA Extraction, Primers was used for molecular identification are lyophilized and was investigated by Macrogen, Korea. Three primers were used for three genes ***16SRNA*** primer sequences are described below.

Table 1: Primers used for the amplification of selected virulence genes that use in study.

Gene	Primer sequence (5' to 3')	Product size/bp
16SRNA	F : TTG TTC ACT TTT TAC CCC TGAA R : CCC TGA CAG CCG TTA GAT ATT	1400 bp

Master mix preparation

The preparation of master mix was done according to manufacturing company that was describing in table (2)

Table (2) showed the volume of each components of master mix solution.

Master mix components	Stock	Unit	Final	Unit	Volume	
					1 Sample	10.05 Samples
Master Mix	2	X	1	X	10	101.000
Forward primer	10	µM	1	µM	1	10.1
Reverse primer	10	µM	1	µM	1	10.1
Nuclease Free Water					5	50.5
DNA	10	ng/µl	10	ng/µl	3	

Identification by (Api 20E) system

The bacterial isolates were further identified by using analytical profile index (API 20E) system according to the procedure suggested by the manufactured company (Bio- Merieux). This system is designed for the performance of 20 standard biochemical tests Lysine decarboxylase (LDC), Ornithine decarboxylase(ODC),citrate utilization(CIT),H2S production, glucose(GLU), Mannitol (MAN), Inositol (INO),Sorbitol (SOR), Rhaminose (RHA),Melibiose(MEL) and Arabinose(ARA),β-galactosidase,(ONPG), Arginine dehydrolase (ADH), Urease(URE), Tryptophane deaminase(TDA), Indol production(IND), Acrtoin production(VP), Gelatinase(GEL), Sucrose(SAC), Amygdaline(AMY) and Cytrochrome-oxidase(OX).

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

RESULTS AND DISCUSSION:

Occurrence of Salmonella spp. by using culture assay

According to the chi-squared analysis, the techniques used to detect Salmonella spp. in chicken showed a significant difference ($p < 0.001$). Table 4-1 shows the distribution of Salmonella spp. isolates by chicken type and isolation method used; the conventional method revealed a total of 21/125 positive cases for Salmonella spp. Salmonella bacteria were isolated from chicken thigh and breast samples collected from five different areas in Karbala city using selective culture media. The results showed variations in isolation rates between the different areas and the chicken species studied. The highest isolation rate was recorded in the Hindiya and Husseiniya areas, with each reaching 25%, while the lowest rate was recorded in the Ain al-Tamr area, where it did not exceed 4.1%. Regarding chicken species, the highest isolation rate was observed in locally slaughtered chicken at 25%, followed by imported chicken at 17.5%, and then randomly slaughtered chicken at 10%. Statistical analysis revealed statistically significant differences between the isolation rates in the different areas and chicken species studied. The identity of the bacterial isolates was confirmed through morphological and biochemical characteristics Table 3, Figure 1

Table1 (3): Ooccurrence of Salmonella spp. in local, imported and random slaughter chicken by culture assay

Location	Local slaughter		Imported chicken		Random slaughter		Total	
Culture	No. of examined	No. positive	No. of examined	No. positive	No. of examined	No. positive	No. of examined	No. of positive
City center	8	2 (25%)	8	0(0%)	8	1 (12.5%)	24	3 (12.5%)
Alhindyia	8	5 (62.5%)	8	1 (12.5%)	8	0 (0)	24	6 (25%)
Al-Hussenyia	8	1 (12.5%)	8	5 (62.5%)	8	0 (0)	24	6 (25%)
Al-Hur	8	1 (12.5%)	8	1(12.5%)	8	3 (37.5%)	24	5 (20.8%)
Ain-Altumor	8	1 (12.5%)	8	0 (0)	8	0	24	1 (4.1%)
Total	40	10 (25%)	40	7 (17.5%)	40	4 (10%)	120	21 (17.5%)
Statistical analysis	X2= 18.22; DF=8; P value= 0.019							

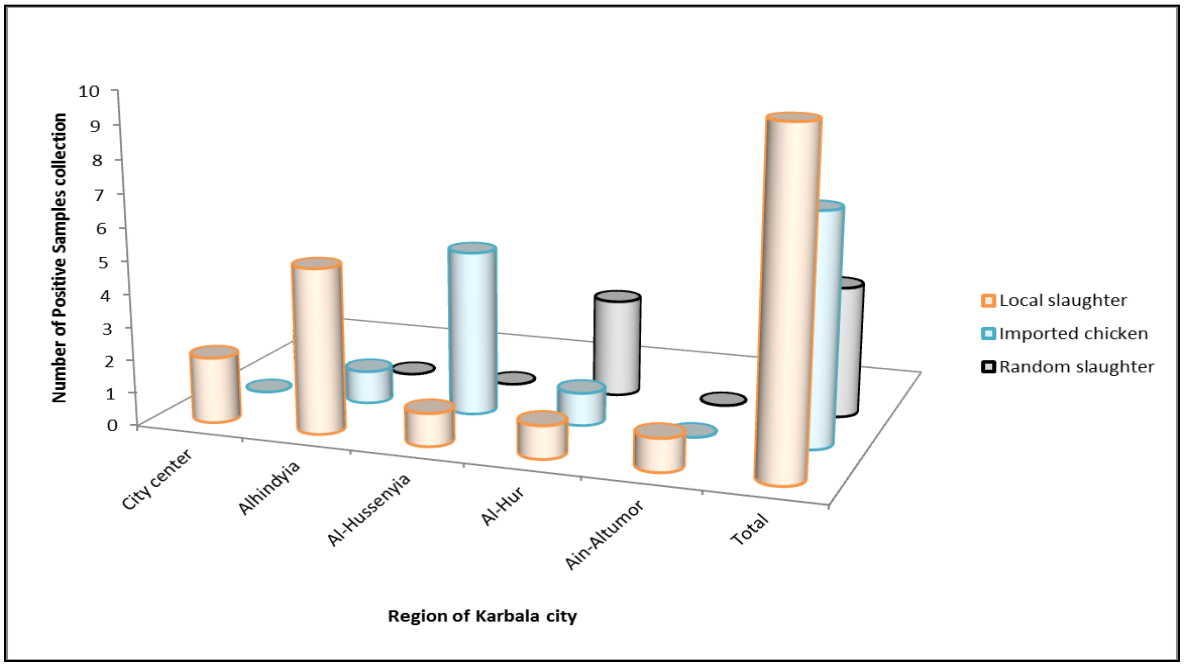

Figure (4-1):Occurrence of Salmonella spp. in local, imported and random slaughter chicken by culture assay

This determination of Salmonella spp. in chicken products using molecular biology techniques, such as PCR, is the first field study conducted in an area such as districts of karbala, which is the main supplier of chicken in Iraq. The chicken production industry is an important sector in the country.

The importance of producing safe products lies in the ability to market them with a certain degree of certainty about their origin and sanitary quality, which translates into a reasonable level of consumer confidence in the products they purchase. Furthermore, it increases the likelihood of successfully accessing increasingly competitive and demanding markets. The presence of pathogenic microorganisms in chicken and the diseases they cause is one of the essential and growing problems in public health, due to their increasing frequency, the emergence of new forms of transmission, the emergence of vulnerable population groups, and the socioeconomic impact they cause (13).

Occurrence of Salmonella spp. by using PCR assay

According to the chi-squared analysis, the techniques used to detect Salmonella spp. in chicken showed non-significant difference ($p > 0.001$). Table 2 shows the distribution of Salmonella spp. isolates by chicken type and isolation method used; the PCR assay revealed a total of 16/125 positive cases for Salmonella spp. Salmonella bacteria were detected in chicken thigh and breast samples collected from five areas in Karbala using PCR technique. The results showed that the total number of positive isolates was 16 out of 120 samples tested (13.3%(. The highest positivity rate was recorded in Al-Hussainiyah district, at 20.83% (five isolates out of 24 samples), followed by Al-Hindiyah district and Al-Hur with the same percentage (4 isolates out of 24 samples). The lowest positivity rate was recorded in Ain Al-Tamr district (4.1%, one isolate out of 24 samples). In terms of chicken type, the highest positivity rate was observed in locally slaughtered chicken (20%, eight isolates out of 40 samples), followed by imported chicken (15%, six isolates out of 40 samples), and then randomly slaughtered chicken (5%, two isolates out of 40 samples). Statistical analysis using the chi-square test showed no statistically significant differences between the different areas (P value = 0.102). It is worth noting that all positive isolates were confirmed through culture and biochemical testing for the Salmonella spp. table 3 figure 2

Table 2: Occurrence of Salmonella spp. in local, imported and random slaughter chicken by PCR assay

Location	Local slaughter		Imported chicken		Random slaughter		Total	
Region	No. of examined	No. positive	No. of examined	No. positive	No. of examined	No. positive	No. of examined	**No. of positive**
City center	8	2 (25%)	8	0 (0%)	8	0 (0%)	24	2 (8.3%)
Alhindyia	8	3 (37.5%)	8	1 (12.5%)	8	0 (0)	24	4 (16.67%)
Al-Hussenyia	8	1 (12.5%)	8	4 (50%)	8	0 (0)	24	5 (20.83%)
Al-Hur	8	1 (12.5%)	8	1 (12.5%)	8	2 (100%)	24	4 (16.67%)
Ain-Altumor	8	1 (12.5%)	8	0 (0)	8	0	24	1 (4.1%)
Total	40	8 (20%)	40	6 (15%)	40	2 (5%)	120	16 (13.3%)
Statistical analysis	X2= 13.267; DF=8; P value= 0.102							

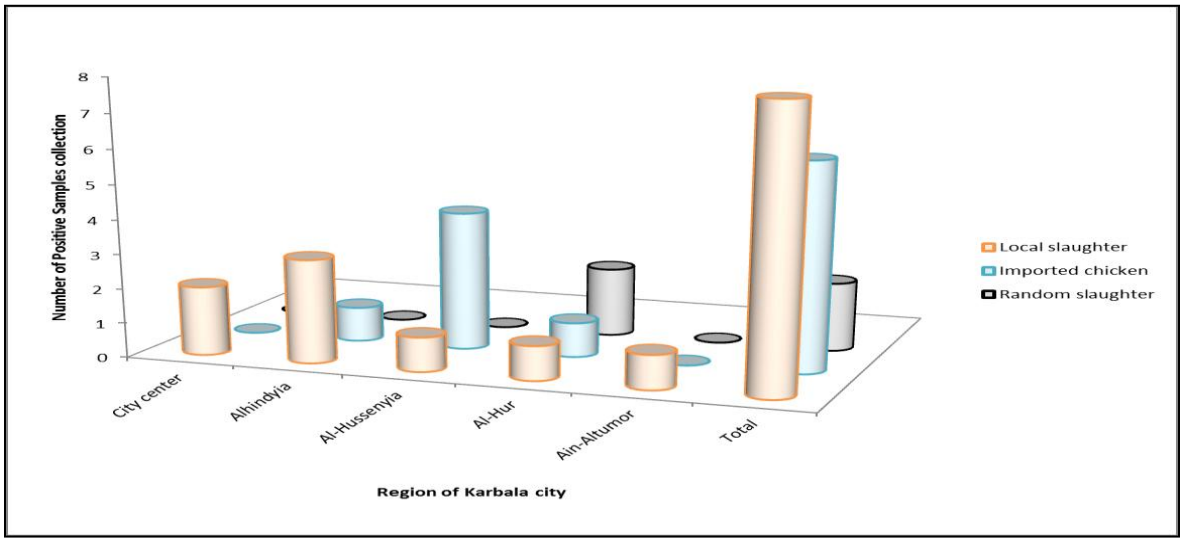

Figure (4): Occurrence of Salmonella spp. in local, imported and random slaughter chicken by PCR assay

Regarding the use of PCR for the rapid detection of Salmonella spp. directly from food, a comparison of the results obtained with the RT-PCR technique with other studies conducted worldwide found that they were lower than those reported by (14), who isolated Salmonella spp. in 25.5%, 11%, and 30.9% of food samples

from the United States and Canada, respectively. However, they are very similar to the results obtained by (15), who isolated Salmonella spp. in 10% of raw meat samples in Japan.

The presence of multiple serovars in a small number of samples implies that control measures such as feed and water hygiene, slaughter sanitation, and breeder stock screening could benefit from enhancement in the local context, in conclusion, the results indicate that S. Typhimurium and S. Enteritidis are the dominant serovars recovered from the sampled chicken sources in Karbala, consistent with international and regional patterns of poultry associated Salmonella contamination. The occasional recovery of rarer serovars suggests that surveillance should remain broad in scope. These findings highlight the need for targeted interventions along the poultry production and supply chain to reduce the risk of zoonotic transmission (16).

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PCR test

All the 21 isolates were subjected to the PCR by using specific primers with amplicon size1400 bp. The results from this molecular technique were promising due to the extraordinarily high rate of Salmonella enterica isolates (16 isolates) containing this genomic region. The electrophoresis results are demonstrated in Figure (5).

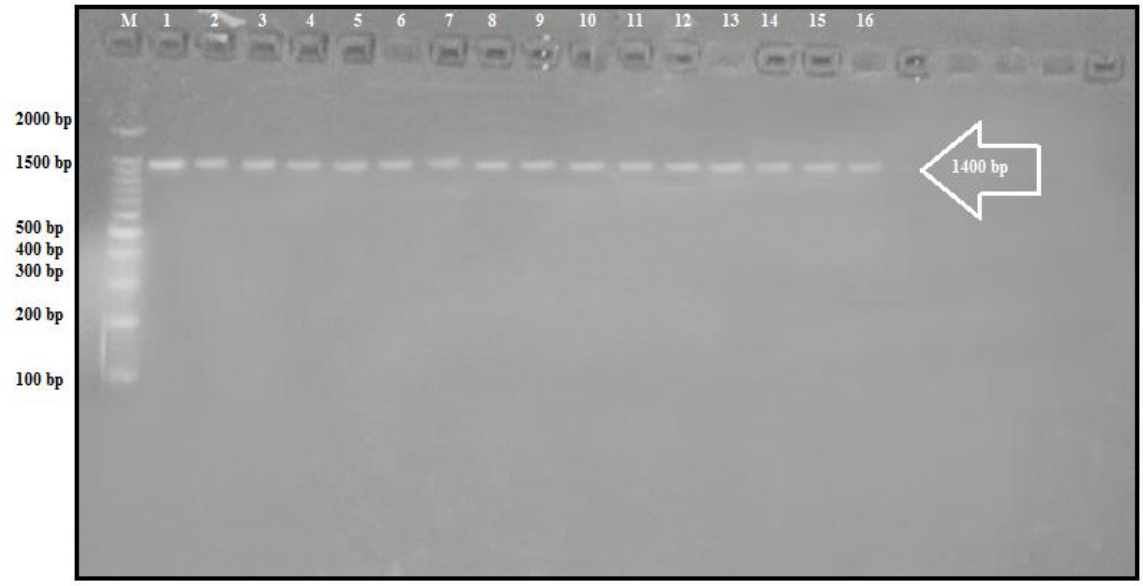

Figure 5: Gel electrophoresis of the ISRs region among Salmonella isolated from poultry samples. Lane (M) DNA Marker (1500 bp), lane 1 to 16 16sRNA gene positive

The 16S ribosomal RNA (16S rRNA) gene is one of the sequences of the rRNA that are critically important in the taxonomy and identification of bacteria, as even salmonella bacteria exhibit an extremely conserved nature that makes the 16S rRNA gene very important in the identifications. The 16 S rRNA gene is common and has served as a molecular marker to prosecute and analyze phylogeny of Salmonella. Sequencing and amplification of this gene have been used to selectively differentiate Salmonella even to its closest members of the family of Enterobacteriaceae. It has been regarded as a gold standard of bacterial identification, especially that has been commonly used in food safety and clinical microbiology laboratories.

Efflux Pump

Efflux pumps were detected in 21 Salmonella isolates using the ethidium bromide–agar cartwheel method (EtBr CW) using ilidium bromide stain as a phenotypic marker. Thus, the efflux pump activity against ethidium bromide was detected in 16 (of 21) Salmonella isolates, which implies the efflux related resistance mechanisms existing highly prevalent in the strains. This observation implies that efflux pumps could play a role in causing antimicrobial resistance in Salmonella of chicken meats and this could be the basis of resistances among various drugs namely; tetracycline, erythromycin and ciprofloxacin etc. Efflux pumps have been found to extrude many different antimicrobial compounds, dyes like the ethidium bromide being a common model of a substrate used to characterize multidrug resistance (16). The prevalence of efflux-positive isolates (76.19%) is compared with other studies which have described the part played by efflux systems, including AcrAB-TolC, in having the ability to resist antibiotic and biocide in Salmonella (Webber et al., 2013). Ethidium bromide efflux was also detected, which implies the participation of the resistance-nodulation-division (RND) family pumps prevalent in Gram-negative bacteria multidrug resistance (17).

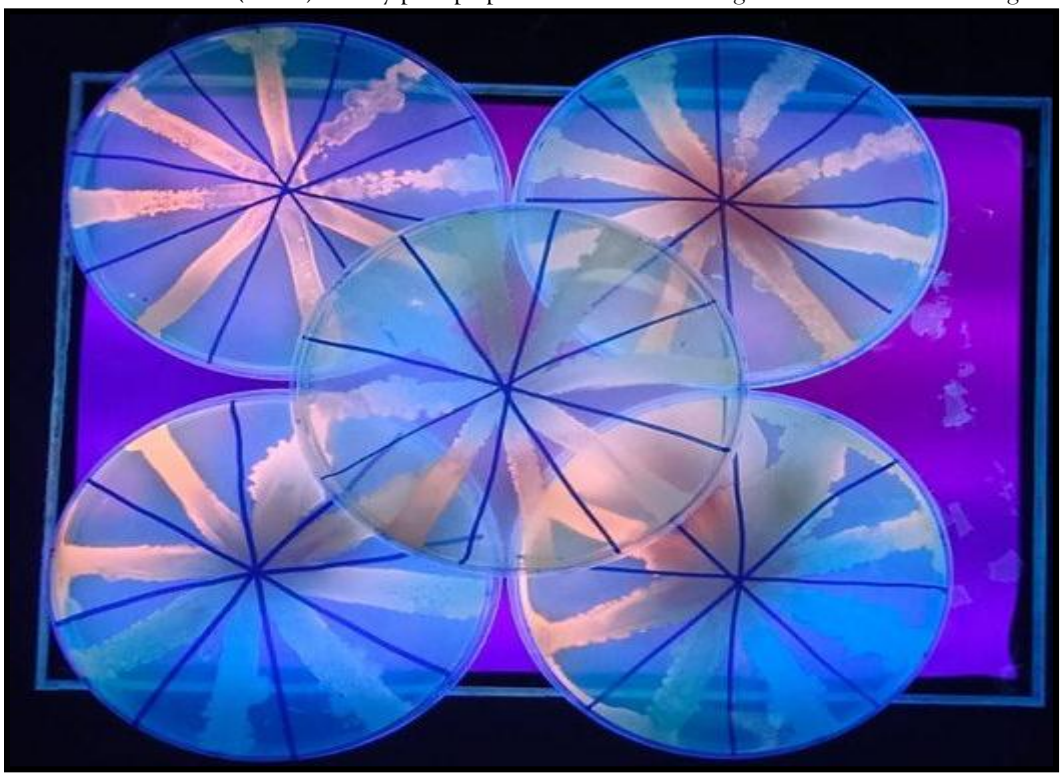

Figure (6) Efflux pupm of Salmonella enteriace agisnt EtBr

The overall meaning of the findings is important, because not only are the efflux pumps associated with developed intrinsic resistance, but also permit higher-level resistance in combination with other mechanisms in cooperation, like target mutation or enzymatic drug deactivation (Sun et al., 2014). The prevalence of efflux in this study can be attributed to the resistance profile to unrelated antibiotics thus the importance of efflux pump inhibitors may serve as an adjuvant treatment to enhance the activity of the administrated antibiotics (18).

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