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Molecular Detection of Quinolones Resistance Gens of *Salmonella* Typhi from Gallbladder of Patients Undergoing to Cholecystectomy in Thi-Qar province/Iraq

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ABSTRACT

Salmonellaenterica serovar Typhi chronically persists within the gallbladder and it may be a predisposing factor for gallbladder diseases. Thus, it is necessary to screen such carriers and understand their distribution within a population with high incidences of gallbladder diseases. This study aimed to focus on determining the actions used by *S.*Typhi to allow chronic carriage in the gallbladder and try to demonstrate that *S.*Typhi may have a causal role in gallbladder diseases. Antibiotic treatment of chronic *S.*Typhi is difficult compared to treatment of acute infection. In the present study, the detection of their antibiotic resistance against 16 antibiotics of different classes showed that all isolates were sensitive to tigraracycline and levofloxacin and whereas all isolates were resistances to ampicillin and cefazolin; the most prevalent pattern included resistance to gentamycin, ciprofloxacin, amikacin, cefoxitin, piperacillin/ tazobactam and cefepime (90.9, 81.8, 63.6, 63.6, 54.5, and 54.5)% respectively.The percentage of multidrug-resistant (MDR) was high, more than (90%) and screening of quinolones-resistance genes (*qnrB* and *qnrS*) among the *S.*Typhi showed *qnrS*- and *qnr-B* gene were identified in (65.4%) and (46.2%) respectively.

Keywords: Gallbladder diseases, *Salmonella* Typhi, Quinolones Resistance

1. Introduction

The gallbladder is one part of the biliary tract; it's a pear-shaped hollow organ; located in a very region on the posterior surface of the right lobe, the length its 7-10 cm with a width around 3 cm and Its work to store and gradually release bile into the digestive system for fat digestion, Bile is emitted into the stomach for digestion from the liver and gallbladder (Halgaonkar *et al*., 2016). The gallbladder diseases are including cholelithiasis (gallstone disease "GS") which is the presence of stones

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within the gallbladder or the duct resulting in pain and discomfort within the abdomen;GS is a significant concern for health services nationwide and is one of the most prevalent conditions in patients with abdominal pain in emergency departments (Dhamnetiya *et al*.,2018).Another disease is cholecystitis that'sinflammation of the gallbladder could be acute or chronic has been caused by biliary tract obstruction due to the presence of gallstones (Wistuba & Gazdar, 2004). In addition to gallbladder carcinoma (GC),the most typical extrahepatic biliary tract malignancy is an aggressive malignancy and the world's mostwidespread biliary tract tumor with the highest occurrence and mortality rates in Northern India (Randi *et al*., 2006).The human's bile is taken into account to be an antimicrobial agentbecause of its properties as detergent in addition to its role in aiding in fat digestion, consequently, that bile prevents the expansion of invading pathogens within the duct thus acting as a defense barrier (Hall-Stoodley & Stoodley, 2009). Although certain bacteria have been evolved resistance to antibacterial effects of bile and they could grow selectively on the media containing bile salts such as (*Salmonella-Shigella* agar and MacConkey agar) .Interestingly, bile is additionally showed to regulate certain bacterial genes expression which is necessary for bile resistance and pathogenesis (Hernandez *et al*., 2012). Different microbiological and molecular methods were used to show the the presence of different bacteria in the gallbladder or hepato-biliary tree suchas *Enterococcus* spp., *Escherichia* spp., *Streptococci* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Staphylococcus* spp., *Pseudomonas* spp. *Acintobacter* spp. and *Salmonella* spp. (Arteta *et al.,* 2017). *Salmonellaenterica* consists of more than 2668 serovar. It can cause disease in both humans and animals (Saleh *et al*., 2016).It will invade the body by infectedfood and water; the epithelium of the small intestine may be inserted within the intestines or penetrated to enter the bloodstream such that it may spread toin the liver, gallbladder, spleen, lungs and other organs (Harvey *et al*., 2013).Thebacteria *Salmonellaenterica* serovar Typhi (*S*.Typhi), the etiologic agent of typhoid fever, causes about 20 million infections each year worldwide (Dougan & Baker, 2014). The clinical symptom of typhoid fever is persistent fever, stomach pain, fatigue and general lethargy. The involvement of distinct protective, as well as offensive virulence factors is consistent with the complex pathogenesis of systemic *Salmonella* infections. As an intracellular human pathogen, these factors contribute to its success and participate in multiple stages of invasion, intracellular reproduction and survival within the host.When *S.* Typhi enters

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the gallbladder and causes an acute infection amid cholecystitis or act as an asymptomatic carrier state to mediate colonization in the gallbladder by this mechanism utilized by the bacterium caused gallbladder abnormalities, especially gallstones (Gunn *et al.*, 2014).Many studies in endemic regions showing a strong link between bacterial colonization in gallbladder and the presence of gallbladder diseases. Furthermore, other studies reveal that chronic *S.*Typhi persistence can be considered as one of the predisposing factors for gallbladder cancer. However, a variety of mechanisms for carcinogenesis in chronic typhoid carriers has been postulated.Colonization of the gallbladder and chronic presence of *S.*Typhi, which is the most effective parameter on the surface of the gallstone, tends to be favored by biofilm formation. The ability to form multilayer biofilm is an essential factor for the virulence of *S.*Typhi and has been shown to promote the survival when they were being exposed to host immunity or antibiotic treatment (Hamilton *et al.*, 2009; Fàbrega &Vila, 2013) .Characterization of the molecular processes involved in the creation of biofilms on biliary stones and the activity of *S.* Typhi remains to be further studied in the promotion of gallbladder inflammation and injury.Clinically prescribed antibiotics are usually ineffective against chronic bacterial infaction of the gallbladder in patients who have gallstones with both *S.* Typhi and cholesterol (Crawford *et al.*, 2008) and the treatment with antibiotics is always unsuccessful in eradicating biofilm-associated bacteria (Zimmerli *et al.*, 2004);once the biofilm is formed, there is an improved susceptibility of individual cells to antimicrobial agents, and antibiotic treatment alone is always insufficient (Hengzhuang *et al.*, 2012). The aims of this study areexplore the antimicrobial resistance profile *in-vitro* and detection of some quinolone resistance genes in *S.* Typhi from gallbladder diseases patients.

2. MATERIALS AND METHODS

2. Screening and Characterization of *S*. Typhi Persisting in Gallbladder of Patients Undergoing Cholecystectomy

2.1 Bacterial cultures

All fresh specimens (bile, gallstones, mucosa and gallbladder sac samples) (n=50) were cultured aerobically by Weigh out 25 g specimens into an Erlenmeyer flask contain 225 ml of buffered peptone water to obtain 1 part sample + 9 part buffer then Mixed and Incubate at 37^{o}C overnight (16-20 hours) then Transfer 1 ml of from the

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inoculated and incubated buffered peptone water with a sterile pipette to 10 ml Tetrathionate broth (Müller-Kaufmann). Incubate at 41.5^{o}C ± 0.5^{o}C overnight (18-24 hours), Spread a 10 µl loop full from the inoculated and incubated Tetrathionate broth on XLD and on BGA agar plates and incubate at 37^{o}C overnight (18-24 hours) and read the XLD plates and BGA plates .*Salmonella* spp. suspect colonies on XLD and BGA agar onto non-selective media, (nutrient agar) plates for biochemical confirmation of *Salmonella* spp.

2.2 Biochemical tests:

The important biochemical tests were conducted these tests include {Triple Sugar Iron (TSI) and Kligler iron (KI), Catalase test, Oxidase test, Lactose fermentation, Urease test, Indole test, Citrate utilization test} (Cappuccino &Sherman, 2017).

2.3 Api-20E system (Analytical profile index for Enterobacteriaceae test)

Api-20E system is used clinically for the rapid identification of the *Salmonella* Typhiisolates this test done according to Leboffe and Piercr, (2005)

2.4VITEK-2 compact system (Pincus, 2010)

Salmonella Typhi which identified by morphological, biochemical tests and Api-20Esystem are subjected to the automated VITEK-2 compact system (VITEK-2 GNID kit) was employed in bacterial diagnosis , screened for their antibiotic resistance against 16 antibiotics of various classes .

2.5Detection of Quinolones Resistance Genes

Quinolones resistance genes (*qnr-B* and *qnr-S)* as in table (1)of*S.*Typhi weredetectedbyMultiplex PCR. Total DNA (2µl) was subjected to multiplex PCR in 23 µl reaction mixture containing 1X PCR Buffer [10 Mm Tris–HCl (pH 8.3), 50Mm KCl ,1.5 Mm $MgCl_2$,200 Mm of each deoxynucleotide triphosphate, 2.5 U of Taq polymerase] ,2µl of each of the four primers and 14 µl of nuclease free water. Amplification was carried out withthermal cycling profile in PCR condition as explained in Table (2) and the products of PCR were visualized by electrophoresis

Table (1): Primers that used for detection of quinolones resistant genes (*qnrB*&*qnrS*) (Bioneer/ Korea)

Gene name		DNA Sequences (5'-3')	Product Size(bp)	Reference

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qnr-B	F	5'-GGMATHGAAATTCGCCACTG -3'	264	(Cattoir *et al.*,2007b)
	R	5'-TTTGCYGYYCGCCAGTCGAA-3'		
qnr-S	F	5'-GCAAGTTCATTGAACAGGCT-3'	428	(Cattoir *et al.*,2007a)
	R	5'-TCTAAACCGTCGAGTTCGGCG-3'		

* H= A or C or T; Y= C or T

Table (2):- Thecondition PCR for quinolones resistant genes amplification

stage		Step	T (˚C)	Time (min)	Number of Cycles
I		Initial Denaturation	95	10	1
II	I	Denaturation	95	1	35
	II	Annealing	54	1	
	III	Extension	72	1	
III		Final Extension	72	10	1

3. RESULTS

A total of 200 samples of gallbladder of GD patients, only 26 samples were positive for *S.*Typhithis is about 13% of patients as show in Figure (1).These were diagnosed by molecular methods to identification genes "*invA*, *viaB* and*H1d*" as illustrated in Figure (1).

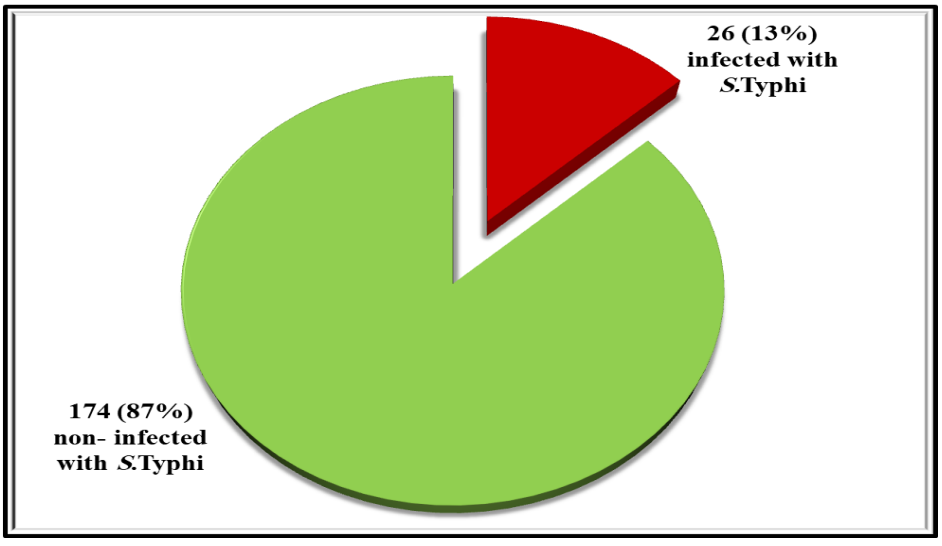

Figure (1): Occurrence of *S.*Typhi in GD patients

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Antimicrobial susceptibility test of the 11 isolates are summarized in Figure (2) .The table indicated that the degree of susceptibility varied and all isolates were sensitive to tigraracycline and levofloxacin .On the other hand all isolates were resistances to ampicillin and cefazolin; the foremost prevalent pattern included resistance to gentamycin ciprofloxacin, amikacin, cefoxitin , pipracillin/tazobactam and cefepime (90.9 , 81.8,63.6 ,63.6 ,54.5 and 54.5) %respectively .The resistances to ceftriaxone, ceftazidime and trimethoprim/ sulfamethoxazole were (45.4%) of isolates, while only (36.36, 36.36 and 18.1) % of isolates were resistances to nitrofurantoin, imipenem and erapenem respectively; while (36.36 27.2 and 9.09) % of isolates were intermediate to nitrofurantion, cefepime and ciprofloxacin respectively.

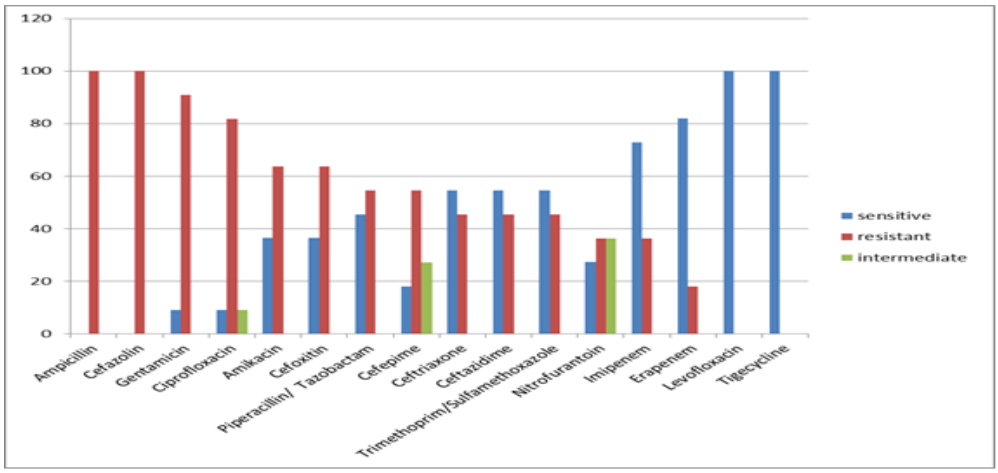

Figure (2): Susceptibility of *S.*Typhi isolates (n=11) to antimicrobial agent

The present study showed an increase in the incidence of multiple drug resistant (MDR), the proportion of multidrug-resistant (MDR) bacteria was high. Ten(90%) isolates considered as multi-drug resistant because the isolates were totally resistance to equal or more than one antibiotic in equal or more than three antimicrobial categories. One isolate was resistant to 11 antibiotics, three isolates were resistant to 10 antibiotics, two isolates were resistant to 9 antibiotics and one isolate was resistant to 8 antibiotics; while three isolates were resistant to 7 antibiotics as shown Figure (3).

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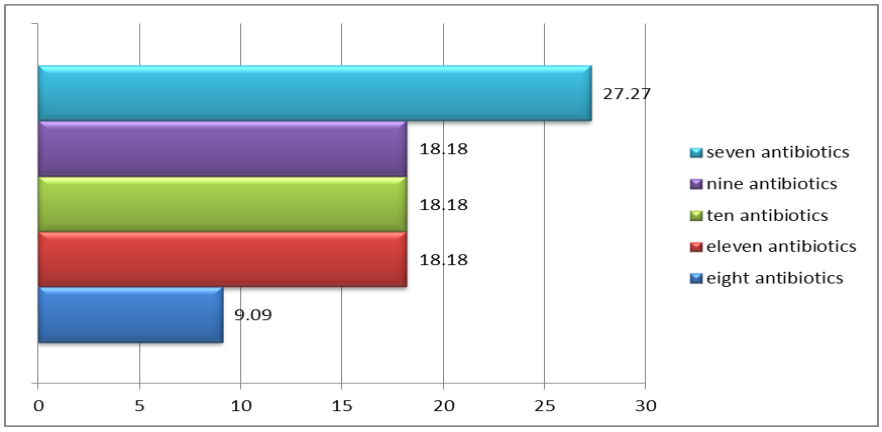

Figure (3):MDR in *S.*Typhi isolates (n=11)

Screening for quinolones-resistance genes (*qnrB* and *qnrS*) among the *S.*Typhi included in this study wasachieved by a multiplex PCR; overall, *qnrS*- gene were identified in 17/26 (65.4 %) of *S.*Typhi. while 12/26(46.2 %) were positive for *qnrB*- gene as appeared in Figure (4).The *qnr-B* gene was absent in half cancerous patients and all a.a.cholecystitis patients, while 52.4% of GS patients contain this gene;

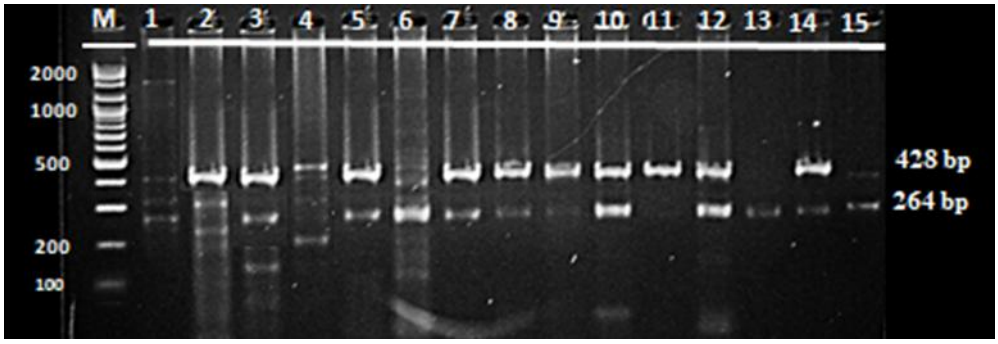

Figure (4): PCR products of the *qnr-B* &*qnr-S* genes of *S.* Typhi, The size of the PCR product is 428 bp and 264 bp respectively. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: Marker DNA ladder (100-2000) bp; (1-15): *S.* Typhi

4. DISCUSSION

Currently, removal of the gallbladder is only cure for chronic *S.*Typhi infection but does not ensure removal of bacteria persisting in ,additional foci within the body and antibiotic treatment of chronic *S.*Typhi persisting in the gallbladder is difficult compared to treatment of acute infection and less than two-thirds of the chronic infections are resolved with prolonged high–dose antibiotic therapy that is associated with side effects like gastric discomfort and gastrointestinal bleeding (Gonzalez-Escobedo *et al.*, 2011). In present research, the resistance to common antibiotics used in the treatment of *S.*Typhi was tested and appeared all the

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chronically persisting isolates of *S.*Typhi obtained from the gallbladder showed resistance ampicillin and cefazolin, gentamycin and high resistance to ciprofloxacin, amikacin, piperacillin/tazobactam, and cefoxitin; these results agree with Al-aarajy, (2020) was found all isolates were resistant to four or more classes of antibiotics as antimicrobials ,Although, AlObaidi*et al.*,(2019) appeared that samples of *S.*Typhi demonstrated total resistance to gentamicin and ciprofloxacin, moderate sensitivity to trimethoprim and complete resistance to amoxicillin and piperacillin.These differences detween studies becauseAlObaidi's isolates were from diarrheal patients in Al-Najaf governorate, whereas, our bacterial isolates were from gallbladder, as the presence of harsh conditions represented by the presence of bile.However, Song *et al.*, (2010) proved the treatment with ampicillin is only effective in patients without gallstones ,while Pratap *et al.,* (2012); showed all the chronically persisting isolates of *S.*Typhi obtained from the gallbladder were resistant to common first-line antibiotics aside from ampicillin, here, our results demonstrated that *S.*Typhi wasbiofilm formation so that was more resistant to ciprofloxacin these findings agree with a study by Parry & Threlfall, (2008) which appeared more resistant to ciprofloxacin in biofilm formation isolates; However, a fluoroquinolone antibiotic is commonly used to treat *Salmonella* infections. By the altering drugs targets, reducing drugs, accumulation, shielding drug targets and enzymatic drug modification several gram-negative bacteria can resist quinolones (McDermott *et al.,* 2003). Also, chromosomal mutations in genes that code for topoisomerase IV and DNA gyrase and genes code for outer membrane and efflux proteins are largely responsible for this resistance, qnr proteins protecting DNA gyrase and topoisomerase-IV from quinolones (Robicsek *et al*., 2006).On a wide scale, Touati *et al*., (2008) determinants of Qnr have been found in North and South America, Europe, Africa and Asia. They've been included in several different Enterobacteriaceae spieces such as *E. coli, Enterobacter spp., Klebsiella spp., Salmonella* spp., *Providencia stuartii,Proteus mirabilis* and *Serratia marcescens* (Cambau *et al*., 2006). According to the present results, percentage of (81.8) % of the isolates was resistant to ciprofloxacin. So that investigated the presence of *qnr* genes among *S.*Typhi were essential and showed there were 17 positive results to *qnrS* and 12 positive results for *qnrB*, while 10 were carry both *qnr* resistance genes. The domination of the *qnrS* and *qnrB* genes in present research is close to the European study by Poirel *et al*., (2006).Similarly, in

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the study by Mohammad, (2017) was found quinolones resistance gene in gram-negative bacteria were high in clinical isolates. In addition, Le Hello *et al.,* (2013) explained that some plasmid-mediated genes which coded for DNA topoisomerase protecting proteins responsible for quinolone resistance in *Salmonella*.The emergence of widespread multidrug resistance (MDR) among *Salmonella* strains could have a significant impact on public health (Parsons *et al.,* 2013).Due to the continuous rise in the MDR in *S.*Typhi especially in carriers state, so that, it is difficult to treat such infections.The present research showed increasing in the incidence of MDR and the proportion of MDR was over (90%). These results resemble to the findings of other researchers like El-Ma'adhidi, (2004) and Misra *et al*., (2005) .Worldwide, Emborg *et al*., (2003) reported during the last decades, the uncontrolled using of antimicrobial agents for growth promotion, treating patient, veterinary fields and prevention in conventional food production have led to the event of antibiotic resistance in *Salmonella*.

Conclusion:-

1. From the analysis of the drug susceptibilities of the isolates, it was seen that all isolates were sensitive to tigraracycline and levofloxacin whereas all isolates showed resistance to ampicillin and cefazolin and high in resistance to gentamycin, ciprofloxacin, amikacin, pipracillin/tazobactam and cefoxitin and more than 90% of all the assessed isolates were MDR.
2. Quinolones resistance rates in *S.*Typhi were high and the most of the resistance isolates harboring *qnr-S* gene

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