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DETECTION OF *SALMONELLA TYPHI* AND HELICOBACTER GENUS IN GALLSTONE PATIENTS

Awatif H. Issa*, Muthena M. Muthena and Adnan Al-Badran

College of Science, Basrah University, Iraq.

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***Correspondence for Author**
Dr. Awatif H. Issa
College of Science,
Basrah University, Iraq.

INTRODUCTION

The bacterial species found in the bile of patients with biliary disease (both gallstones and cholangitis) indicate that the intestinal flora is a main source of bacterobilia. *Escherichia coli (E.coli), Streptococci* or *Enterococci, Enterobacter, Klebsiella, Pseudomonas,* and *Proteus* are the species most frequently isolated from aerobic cultures. *Clostridium* and*Bacteroides* species are often found in anaerobic isolates. In about half or more of the isolates from bile, a mixed infection is found. (Tabata& Nakayama, 1981; Vitetta*et al.*, 1989).*Propionibacterium* species were isolated from bile in low frequency (Brismar*et al.*, 1986). Actinomycetes species have been cultured from the center of human gallstones (Rains*et al.*, 1960). Recently, there has been a great deal of interest in a possible connection between the gastric pathogen *Helicobacter pylori* and gallstones (Farshad*et al*., 2004; Maurer *et al*., 2006; Silva*et al.*, 2003). Interestingly, growth of this organism is inhibited by bile salts both in vivo and in vitro, and chemotactic assays show that bile salts actually repel the organism (Maurer *et al*., 2006). Findings that contradict the ability of *H. pylori* to colonize a healthy gallbladder,nonetheless, a number of groups claimed to have identified *H. pylori* DNA in biliary tissue and gallstones, however there are several problems with these studies that some samples were collected at endoscopic retrograde cholangiopancreatography(Silva*et al.*, 2003).Because *H. pylori* colonizes the gastric mucosa of more than half the world's population, the statistical chances for sample contamination by gastric *H. pylori* are high, Additionally, 16S ribosomal RNA genus-specific primers are commonly used to identify these organisms in polymerase chain reaction (PCR) analysis, despite the fact that these primer sets can amplify other non–*H. pylori*helicobacters(Maurer *et al*., 2006).

These organisms could invade the biliary tree following alterations in the biliary microenvironment and especially biliary dysmotility. Supporting this hypothesis is a recent

study showing that *H. pylori* DNA was present in gallbladder bile of patients with chronic cholecystitis, consistent with obstruction, but not in asymptomatic patients with gallstones (Farshad*et al*., 2004).

Since the discovery of *Helicobacter pylori* in 1983 AD, more than 25 additional Helicobacter species have been isolated. Among them *Helicobacter hepaticus* was reported by peer-reviewed literature in 1994 AD. A microorganism resembling *Helicobacterpylori* was detected incidentally on pathologic specimen of the gallbladder mucosa (Fox *et al*., 1999; Leong *et al*., 2002) and has reported a possible association of *Helicobacter hepaticus* species with gallbladder disease. Such an action is presumably enhanced by the presence of gallstone. Furthermore, bile resistant Helicobacter species are found in bile from patients with chronic cholecystitis and their presence is considered to be a risk factor for malignancy (Fox *et al*., 1999).

This organism was first identified in mice in 1992 (Fox *et al*., 1994) and subsequently characterized by ultra-structural morphological examination, biochemical characteristics and 16 S rRNA genes sequence determined to be a new species related to *helicobacter* genus, it was given the name *Helicobacter hepaticus* (Rice , 1995).

In Pradhan& Dali study, 82% of the gallbladder specimens were found to have *Helicobacter hepaticus* infection. This might be the leading cause for the precipitation of the cholesterol that ultimately forms the gallstone (Pradhan& Dali, 2004).

In addition to the gastric pathogen *H. pylori* and *Helicobacter hepaticus*, a variety of enterohepatichelicobacters (eg, *Helicobacter bilis*) exist. These reside in the small and large intestines and canalicular spaces of the liver of experimentally and naturally infected animals, including humans (Solnick&Schauer, 2001). DNA from these organisms was detected in nonwhite patients with chronic cholecystitis, gallstones, and malignant biliary tract diseases (Fox *et al*., 1999; Matsukura*et al*., 2002).

One of the classic bacterial pathogens of the biliary tree in humans is *Salmonella enterica*serovar*typhi*, which causes typhoid fever (Dutta*et al*., 2000). This organism crosses the intestinal epithelial barrier, invades macrophages, and spreads systemically (Prouty*et al*., 2002). After colonizing the liver, the organism can be shed into the gallbladder and chronically colonize the gallbladder wall (3%–5% of those infected). Although chronic

colonization with *S.typhi* is associated with gallstones and gallbladder cancer (Dutta*et al*., 2000).

It is not clear whether this organism contributes to stone formation or alternatively if the presence of gallstones promotes chronic colonization. Recent evidence indicates that *S. typhi* forms bacterial biofilms on the surface of gallstones. This biofilm formation would allow for chronic colonization and protect the organism during antibiotic treatment. Biofilm formation is dependent on the presence of bile because organisms cultured without bile do not readily form biofilms (Prouty*et al*., 2002). This indicates that this organism is exquisitely adapted to survival in a gallbladder that contains gallstones. Furthermore, biofilm formation is reduced when pebbles or glass beads are utilized instead of gallstones, indicating that both bile and gallstones are essential for biofilm formation. Moreover, colonization in the presence of gallstones requires expression of several bacterial genetic components (Prouty*et al*., 2002; Prouty&Gunn, 2003).

These data argue that gallstones promote chronic colonization with *S.typhi* rather than *S.typhi*–promoting gallstones. Regardless of whether gallstones promote colonization or vice versa, the concomitant presence of gallstones and *S. typhi* markedly promotes the risk of gallbladder cancer, making the interaction between *S.typhi* and gallstones an important topic of clinical interest (Dutta *et al*., 2000; Prouty*et al*., 2002).

MATERIALS AND METHODS

Samples collection

The gallbladder samples (52 tissue sample, 20 bile sample, 48 stone sample and 52 blood sample) were collected from 52 patients with age ranged from (11-66) years submitted to the cholecystectomy operation in Al-sader Teaching Hospital in Basra province through the period from Nov. 2011 to Feb. 2012. The appropriate procedures for specimens collection for bacteriological and immunological analysis are described below. Those specimens were collected in proper ways to avoid any possible contamination (Colleet*al.*, 1996).

Tissue, Bile, Stone samples

Specimens were collected from each patients submitted to the cholecystectomy operation in sterilized plain tube containing PBS for bacterial culture.

Direct DNA extraction from specimens (Tissue, Bile & Stone)

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company (Promega) as follow:

-Prepare specimens

- Tissue: The fresh or thawed tissue 10–20mg was added to 600µl of chilled Nuclei Lysis Solution and homogenized for 10 seconds. Incubated at 65°C for 30 minutes.
- Bile: The bile centrifuged at 15,000 × g for 10 seconds. The bile was washed with PBS, vortexed and then added 600µl of Nuclei Lysis Solution and mixed by pipetting.
- Stone: The stone 10–20mg was added to 600µl of chilled Nuclei Lysis Solution and homogenized for 10 seconds. Incubated at 65°C for 30 minutes.
- A volume of 3 µl of RNase solution was added to the specimen lysate. Invert the tube 5 times to mix.
- The tube incubated at 37^{o}C for 30 min. cool the specimen to room temperature.
- A volume of 200µl of protein precipitation solution was added to specimen treated lysate. Vortexed at high speed for 20 seconds for mixing.
- The specimen was incubated in ice for 5 min.
- The tube was centrifuged at 15.000 xg for 4 min.
- The supernatant containing the DNA was transferred to a clean tube containing 600µl of room temperature isopropanol.
- The tube was mixed gently by inversion.
- The tube was centrifuged at 15.000 xg for 1min.
- The supernatant were poured off carefully and drain the tube on clean absorbent paper.
- A volume of 600 µl of room temperature 70% ethanol was added and gently mixed the tube several times to wash the DNA pellet.
- The tube was centrifuged at 15.000 xg for 1min. carefully aspirate the ethanol.
- The tube was drained on clean absorbent paper and the pelletexposured to air-dry for 15 min.
- A volume of 100µl of DNA rehydration solution was added to the tube and the DNA was rehydrated by incubation at 4^{o}C for overnight.
- The DNA was stored at -20 ^{o}C.

Detection of genomic DNA by agarose gel electrophoresis

Genomic DNA was determined by agarose gel electrophoresis (Sambrook and Russell, 2001).

Agarose gel was prepared by dissolving 0.2 gm of agarose powder in 25 ml of TBE buffer (pH: 8) by heating to boiling, after cooling to 50^{o}C, 0.5 mg/ml of ethidium bromide was added.

The comb was fixed at one end of the tray to form wells for loading DNA specimen. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min.

The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 10µl of DNA specimen (7 µl DNA + 3 µl Bromophenol blue) was transferred into the wells in agarose gel. The electric current was allowed at 60 volt for 60min. UV transilluminater was used for the observation of DNA bands.

Detection of bacterial infections by PCR

The presence of bacteria in the clinical specimens was detected by using PCR in order to amplify universal bacterial 16s rRNA gene, Helicobacter genus 16s rRNA gene & Salmonella entericaserovartyphi 16s rRNA gene.

A- Primers: A bacterial infection was detected by using the following primers which listed in Table (1).

Table (1): Primers used in PCR amplification.

Gene	Primer type	Primer sequence (5′-3′)	Size of product bp	Reference
Universal bacterial 16s rRNA	B 2 7 F U1492R	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-GGTTACCTTGTTACGACTT-3′	1500	Miyoshi *et al.*, 2005
Helicobacter genus16s rRNA	C 9 7 F C98R	5'-GCTATGACGGGTATCC-3′ 5'-GATTTTACCCCTACACCA-3′	400	Fukuda *et al.*, 2002
Salmonellatyphi16s rRNA	S R 1 F SR2R	5'- AGT TTG ATC CTG GCT CAG-3' 5'- AGT ACT TTA CAA CCC GAA GG-3'	423	Massi*et al.*, 2005

B- The reaction mixture

Amplification of DNA for universal 16SrRNA (B27f , U1492r) was carried out in a final volume of 20 µl (Table 2) containing the following:-

Table 2: The reaction mixture of universal 16SrRNA.

S. No	Contents of reaction mixture	Volume
1.	master mix	5 µl
2.	Upstream (B27) primer	1µl(10pmol)
3.	Downstream (U1492) primer	1 µl(10pmol)
4.	DNA template	5 µl
5.	Nuclease free water	8 µl
Total volume		20 µl

Amplification of DNA for *Helicobacter genus16s rRNA* (C97F, C98R) was carried out in a final volume of 20 µl (Table 3) containing the following:-

Table 3: Contents of the reaction mixture for *Helicobacter genus16s rRNA* primer amplification.

No.	Contents of reaction mixture	Volume
1.	master mix	5 µl
2.	Upstream (C97) primer	1µl(100pmol)
3.	Downstream (C98) primer	1µl(100pmol)
4.	DNA template	5 µl
5.	Nuclease free water	8 µl
Total volume		20 µl

Amplification of DNA for Salmonella typhi*16s rRNA* (SR1, SR2) was carried out in a final volume of 20 µl (Table 4) containing the following:-

Table 4: Contents of the reaction mixture for Salmonella typhi*16s rRNA* primer amplification.

No.	Contents of reaction mixture	Volume
1.	master mix	5 µl
2.	Upstream (SR1) primer	1µl(10pmol)
3.	Downstream (SR2) primer	1 µl(10pmol)
4.	DNA template	5 µl
5.	Nuclease free water	8 µl
Total volume		20 µl

C- Thermal cycling conditions

The reaction was performed according to Miyoshi *et al*., 2005 for universal *16s rRNA* primer, Fukuda *et al.*, 2002 for *Helicobacter genus16s rRNA*primer , Massi*et al.*, 2005*Salmonella typhi16s rRNA*primer in a PCR thermal cycler apparatus , after several trials , the following programs was adopted as illustrated in table (5) , table (6) and table (7) :-

Table (5): The cycling conditions of universal *16s rRNA* primer.

Steps	Temperature	Time	No. of cycles
Initial denaturation	92 ºC	2 min	1
Denaturation	94 ºC	30 sec	30
Annealing	51 ºC	45 sec	
Extension	72 ºC	1.5 min	
Final extension	72 ºC	5 min	1

Table (6): The cycling conditions of specific *Helicobacter genus16s rRNA*primer.

Steps	Temperature	Time	No. of cycles
Initial denaturation	95 ºC	5 min	1
Denaturation	94 ºC	1 min	40
Annealing	55 ºC	30 sec	
Extension	72 ºC	1 min	
Final extension	72 ºC	5 min	1

Table (7): The cycling conditions of specific *Salmonella typhi16s rRNA*primer.

Steps	Temperature	Time	No. of cycles
Initial denaturation	95 ºC	5 min	1
Denaturation	94 ºC	1 min	40
Annealing	55 ºC	1 min	
Extension	72 ºC	2 min	
Final extension	72 ºC	10 min	1

Detection of amplified products by agarose gel electrophoresis

PCR amplification products were determined by agarose gel electrophoresis (Sambrook and Russell, 2001).

Agarose gel was prepared by dissolving 0.5 gm of agarose powder in 25 ml of TBE buffer (pH: 8) by heating to boiling, after cooling to 50^{o}C, 0.5 mg/ml of ethidium bromide was added.

The comb was fixed at one end of the tray to form wells for loading specimen. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 10µl of PCR product was transferred into the wells in agarose gel, and in one well we put the 5µl DNAladder.

The electric current was allowed at 60 volt for 60min. UV transilluminater was used for the observation of DNA bands.

RESULTS AND DISCUSSION

DNA was extracted from 120 gallbladder specimen and Nano drop was performed to estimate DNA concentrations Table (8). Agarose gel electrophoresis was performed to detect the presence of extracted DNA in gallbladder specimens Figure (1).

Table (8): The concentrations of extracted DNA obtained by promega extraction kit from 120 gallbladder specimens obtained from 52 patients under cholecystectomy operation.

Gallbladder specimens type	No. of extracted DNA specimens	DNA concentration mean µg/ml
Tissue specimens	52	804.59
Bile specimens	20	267.98
Stone specimens	48	244.67
Total specimens	120	439.08

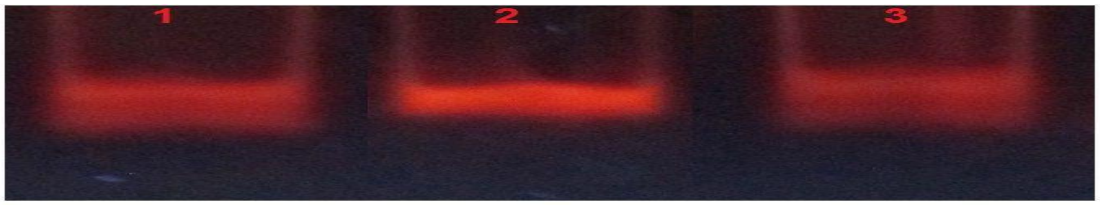

Figure (1): Agarose (0.8%) gel electrophoresis patterns show DNA extraction bands from gallbladder specimens lanes (1-3).

Polymerase Chain Reaction (PCR) technique

PCR technique was used for detection of bacterial genes in gallbladder specimens.

Molecular base of universal 16S rRNA gene detection by PCR

The extracted DNA from 120 gallbladder specimens (52 tissue specimen , 48 stone specimen and 20 bile specimen) were subjected to PCR for amplifying bacterial universal 16SrRNA gene which detect any bacterial species DNA in gallbladder specimens. PCR products for the universal 16S rRNA based primers gave a sharp band on agarose gel corresponding to 1500 bp product when compared to the molecular ladder (100-2000 base pair) Figure (2). The results of universal 16S rRNA gene detection in gallbladder specimens showed 69 (57.50%) positive gallbladder specimens and the stone specimens appear 60.42% positive gallbladder specimens for universal 16S rRNA gene that more than other types of gallbladder specimens Table (9). The frequency of bacterial DNA detected with PCR in this study is much higher

and differs strongly from the culture results. The striking difference between cultural and molecular genetic findings is because of the differences in methodology (Swidsinski*et al*., 1995).

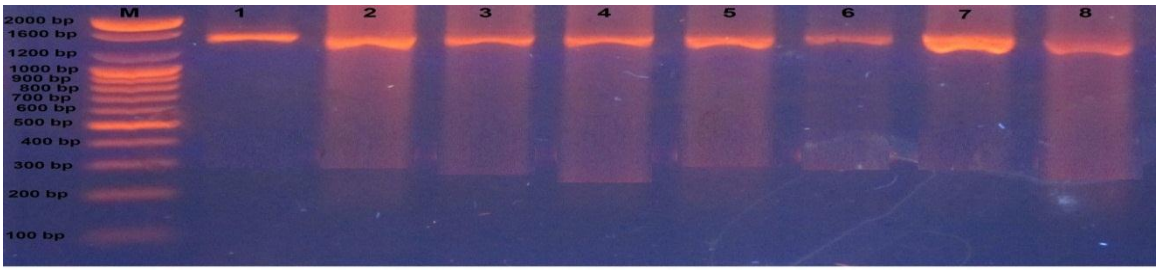

Figure (2): Agarose (2%) gel electrophoresis patterns show PCR amplified products of universal 16s rRNA gene lane M: 100-2000 bp DNA ladder, lanes (1-8): universal 16s rRNA bands of gallbladder specimens.

Table (9): The results of universal 16S rRNA gene in 120 gallbladder specimens obtained from 52 patients under cholecystectomy operation.

Gallbladder specimens type	No. of specimens	No. of positive specimens	Percent of positive growths
Tissue specimens	52	28	53.84 %
Bile specimens	20	12	60 %
Stone specimens	48	29	60.42 %
Total specimens	120	69	57.5 %

Molecular base of *Salmonella typhi* 16S rRNA gene detection by PCR

The extracted DNA from 69 positive gallbladder specimens for universal 16S rRNA gene (28 tissue specimen, 29 stone specimen and 12 bile specimen) was subjected to PCR for amplifying bacterial *Salmonella typhi16S rRNA* gene which detect *Salmonella typhi*species DNA in gallbladder specimens. PCR products for the *Salmonella typhi*16S rRNAbased primers gave a sharp band on agarose gel corresponding to 428 bp product when compared to the molecular ladder (100-2000 base pair) Figure (3).

The results of *Salmonella typhi* 16S rRNA gene detection in gallbladder specimens showed 62 (89.86%) positive gallbladder specimens from positive gallbladder specimens for universal 16S rRNA gene and the stone specimens revealed 93.10% positive gallbladder specimens for *Salmonella typhi* 16S rRNA gene which is more than other types of gallbladder specimens Table (10).

There is an old controversy about the association between typhoid fever or salmonella infection of gallbladder and the formation of the gallstones, one theory states that germs will act as a nidus for stone formation, while the other supports the idea that cholelithiasis will

make gallbladder more prone to salmonella infection (Saunders *et al.*, 1990). The latter is supported by the fact that chronic biliary carriers of *salmonellatyphi* and paratyphi almost always have abnormal gallbladder, mainly the presence of stones (Christie, 1987). *Salmonella typhi* can colonize the gallbladder and persist in an asymptomatic carrier state that is frequently associated with the presence of gallstones, gallbladder typhoid carriage is often a result of biofilm formation on gallstone surfaces (Crawford *et al.*, 2010). Intracellular replication of Salmonella occurs in gallbladder epithelial cells and Salmonella are resistant to physiological bile and can use this harsh environment for efficient extracellular replication. This supports the notion that bacterial bile resistance is an important trait for bacterial survival and persistence (Menendez *et al.*, 2009). Bile resistance is a complex phenomenon, as indicated by the observation that Salmonella cultures can be adapted to resist extremely high bile concentrations by previous exposure to a sub lethal dose (Van Velkinburgh& Gunn, 1999). Prouty*et al.*, (2002) study has shown that bile induces the formation of Salmonella biofilms on the surface of gallstones. The gallbladder is the niche for chronic Salmonella infections, which are in turn associated with gallstone formation and development of hepatobiliary carcinomas (Dutta*et al.*,2000). In Crawford *et al.*, (2008) study identified a critical Exopolysaccharide (the O-antigen (O-ag) capsule) required for biofilm formation specifically on gallstone. It was detected in the extracellular matrix of Salmonella bile-induced biofilms on gallstones and hypothesized that bile induction of the O-ag capsule has evolved to aid chronic carriage of *Salmonella typhi* specifically in the gallbladder environment. These results agreed with Vaishnavi*et al.*, (2005).

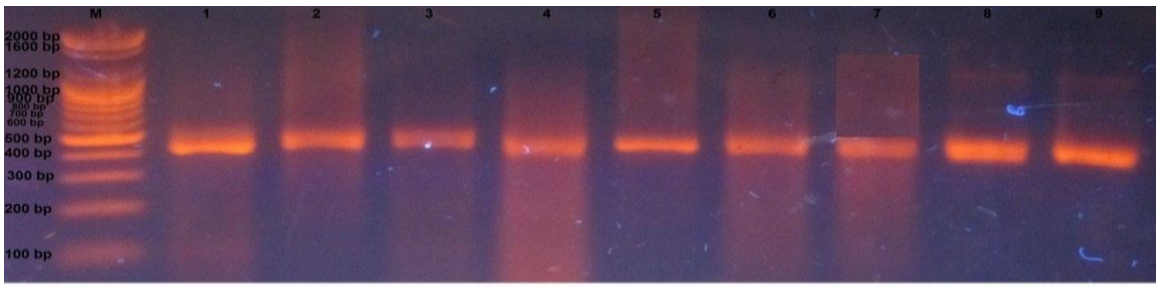

Figure 3: Agarose (2%) gel electrophoresis patterns show PCR amplified products of *Salmonella typhi16s rRNA* gene lane M: 100-2000 bp DNA ladder, lanes (1-9): *Salmonella typhi16s rRNA* bands of gallbladder specimens.

Table 10: The results of *Salmonella typhi16SrRNA* gene in different types of positive gallbladder specimens for universal *16S rRNA* gene.

Gallbladder specimens type	No. of specimens	No. of positive specimens	Percent of positive growths

Tissue specimens	28	26	92.86%
Bile specimens	12	9	75%
Stone specimens	29	27	93.1%
Total specimens	69	62	89.86%

Molecular base of Helicobacter genus 16S rRNA gene detection by PCR

The extracted DNA from 69 positive gallbladder specimens for universal *16SrRNA* gene was subjected to PCR for amplifying bacterial Helicobactergenus*16S rRNA* gene which detect Helicobacterspp.DNA in gallbladder specimens. PCR products for the *Helicobacter genus 16S rRNA*based primers gave a sharp band on agarose gel corresponding to 400 bp product when compared to the molecular ladder (100-2000 base pair) Figure (4).

The results of Helicobacter genus*16S rRNA* gene detection in gallbladder specimens showed 25 (36.23%) positive gallbladder specimens from positive gallbladder specimens for universal *16S rRNA* gene and the bile specimens appear 75% positive gallbladder specimens for Helicobacter genus*16S rRNA* gene more than other types of gallbladder specimensTable (11). It is also possible that after entrance of sensitive Helicobacter spp. to the bile system it loses its viability in the presence of salt components of bile but its DNA and antigens persist there for a longer time, of course the inhibitory effects of bile alone may inhibit the growth of the organisms, producing a false negative result in bacterial culture (Farshad*et al*., 2006). Different Helicobacter species can be present in the human biliary tree but that the number of microorganisms can differ according to the species, with several intestinal Helicobacter species being present in higher numbers than *H.pylori*, this difference may be explained by the fact that intestinal Helicobacter species (such as *H. hepaticus,H. rappini, H. bilis, H.canis, H. cholecystus,* and *H. pullorum*) are resistant to bile , a property that may confer protection against the deleterious effects of bile and adapt them better to the hepatobiliary milieu (Silva *et al*., 2003). *H. pylori* are sensitive to bile salts, especially the unconjugated bile salts that exert poisonous effect on *H. pylori* and it was cannot live in an environment with bile salts. a kind of *H. pylori* that can resist bile salts exists. Bile regurgitation may play a role in selecting *H. pylori* so that *H. pylori* resistant to bile salts can survive. Thus, *H. pylori* that resist the bile salts and survive under basic conditions can enter the gallbladder through the common passage (Chen *et al*., 2007). It has been proposed that the presence of *H. pylori* in bile may represent an increased risk of gallstone formation. A possible consequence of colonization by Helicobacter spp. is a chronic inflammation in the gallbladder mucosa. This inflammation may impair gallbladder mucosa acid secretion and

acidification of the contents, reducing the solubility of calcium salts in gallbladder bile and increasing the risk of their precipitation in the lumen (Ghazal *et al*., 2011). These results agreed with Silva *et al*. (2003) , Lee *et al*. (2010) and Sabbaghian*et al*. (2010) that detected Helicobacter genusDNA in gallbladder specimens and reported that the bile specimens appear positive gallbladder specimens for Helicobacter genus*16S rRNA* gene more than other types of gallbladder specimens.

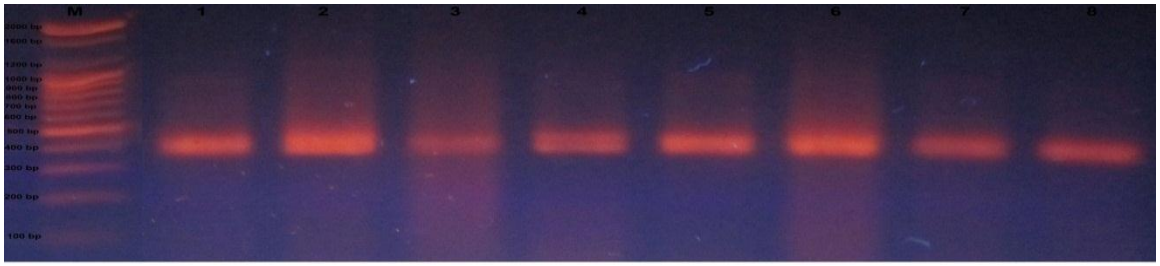

Figure (4): Agarose (2%) gel electrophoresis patterns show PCR amplified products of Helicobacter genus*16s rRNA* gene lane M: 100-2000 bp DNA ladder, lanes (1-8): Helicobacter genus*16s rRNA* bands of gallbladder specimens.

Table 11: The results of Helicobacter genus16S rRNA gene in different types of positive gallbladder specimens for universal 16S rRNA gene.

Gallbladder specimens type	No. of specimens	No. of positive specimens	Percent of positive growths
Tissue specimens	28	8	28.57%
Bile specimens	12	9	75%
Stone specimens	29	8	27.59%
Total specimens	69	25	36.23

The positive gallbladder specimens of Helicobacter genus16S rRNA and *Salmonella typhi*16S rRNA genes were 23 (11.59%) gallbladder specimens from positive gallbladder specimens for universal 16S rRNA gene (8 (28.57%) tissue specimen , 8 (66.67%) bile specimen and 7 (24.14%) stone specimen). The presence of the Helicobacter genus and *Salmonella typhi*bacteria in gallbladder specimens may represent a cooperative role in the pathogenicity of gallstone disease.

DNA extraction from isolated bacteria from gallbladder specimens

The extracted DNA from 83 bacterial isolates obtained from gallbladder specimens was subjected to PCR for amplifying bacterial *Salmonella typhi16S rRNA* gene and

Helicobactergenus*16S rRNA* gene. The results did not record any presence of these genes in extracted DNA of isolated bacteria from gallbladder specimens.

CONCLUSION

- The presence of *Salmonella typhi*and Helicobacter spp. DNA in gallbladder specimens supported the presence of bacterial causes for gallstone.

Recommendation

- Studying the relationship of *Salmonella typhi* in gallstone formation.
- Studying the pathogenicity of *Helicobacter* spp. in gallstone formation.
- Detection of *Helicobacter* genus species and genes of Cag and Vac in gallstone patients.

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