

# Histopathological study of Gallbladder diseases and Molecular Detection of genotoxins of Salmonella Typhi from Gallbladder of Patients Undergoing to Gallbladder Cholecystectomy in Thi-Qar province/Iraq

Zaman K. Hanan<sup>1</sup>, Ezat H. Mezal<sup>2</sup>, Manal B.Saleh<sup>3</sup>,Hazar Shaker Saleh<sup>4</sup>

<sup>1,3</sup> Department of Biology - Collage of science – University of Thi-Qar. Iraq

<sup>2</sup>Collage of Nursing – University of Thi-Qar. Iraq

<sup>4</sup> Department of biology, college of education for pure science- University of Thi- Qar . Iraq

Corresponding email: zaman.K\_bio@sci.utq.edu.iq

**Abstract:** *This study was carried out in high graduated laboratories in department of biology, college of science / university of Thi-Qar, during the period from December 2019 to October 2021. Gallbladder Undergoing to Cholecystectomy were 200 cases were collected and diagnosed in AL-Hussein teaching, Al-Amel Hospitals and Noor Al-Hussein and Ibn-Al- Baitar laboratories in Thi-Qar Province /Iraq and 50 samples of normal gallbladder from forensic as control during the amount from December 2019 to July 2020 from both sexes and different age. After cholecystectomy is done to gallbladder; it's put in containers which contain Neutral Buffered Formalin 10% for Histopathological classification ;The results of histological study appeared that there were the most common diseases were Cholecystitis in patient were 79.0 %, and in control were 5.6%. While the less common disease were cancer in patient were 4.2 %, and in control were 0.00%. A total of 11 isolates of Salmonella Typhi which identified by conventional biochemical test and API 20- E were subjected to DNA extraction , 50 of DNA extracted from tissue in formalin and 100 of DNA extracted from the formalin-fixe paraffin-embedded gallbladder tissue were subjected to PCR assay for presence of invA, viaB and H1d genes. The prevalence of Salmonella Typhi among patients infected with Cancer, Cholecystitis, Galls stones, the result indicate among 200 patient 26 of them infected with Salmonella Typhi with percentage (13.06%). A positive results have seen in 26(100%) of sample subjected to PCR assay for presence of invA, viaB and H1d genes. Tree genes were selected for Screening of Salmonella Typhi producing toxins by Multiplex PCR; these genes are cdtB ,pltA and pltB . The results indicated all toxin genes were isolated from S. Typhi of patients with gallstones, while non CdtB isolated from bacteria in patients with Cholecystitis and 84,6% of S.Typhi contain pltA and pltB genes*

**Keywords:** *Histopathological study, Gallbladder diseases, Salmonella Typhi, genotoxins*

## 1. INTRODUCTION

Gallbladder is a pear-shape organ to digestive storage; it's located below the liver and upper right side of the abdomen; in adult, gallbladder is between (7 to 10 cm) long, with a capacity of up (50 ml) (1). its working for store and gradually release bile for fats digestion(2).Histopathological analysis appeared that gallbladder consists of three layers :- mucosa (innermost), muscularis and adventitia or serosa (outermost)(3)in addition to that it is divided into three regions: The fundus, the body and the neck (4). gallbladder Diseases are common and costly and include the following:- Cholelithiasis is the presence of gallstones in gallbladder or in the bile duct; Cholecystitis is the inflammation of gallbladder that caused by obstruction of the biliary tract and may be acute, chronic, or acute superimposed on chronic; and almost occurs in association with presence the gallstones ; the third disease is Carcinoma of the gall bladder ; it is the fifth most common cancer of gastrointestinal tract (5); it's one of the commonest biliary tract carcinomas (BTC) (6), and has established endemicity in certain countries . Different microbiological and molecular methods have been showed the presence of different bacteria in gallbladder or in hepato-biliary tree such as *Escherichia* spp., *Enterococcus* spp. *Streptococci* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Citrobacter* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Salmonella* spp. and *Acinetobacter* spp. (7). *Salmonella enterica* consists of more than 2668 serovar. It can cause the disease in both human and animals (8). Typhoid fever is one of the most severe *Salmonella* infections, which is caused diarrhea and systemic diseases. Typhoid fever or enteric fever, caused primarily by *Salmonella* Typhi (*S. Typhi*), is a human systemic disease that is responsible for an estimated 21 million new infections per year resulting in approximately 200,000 deaths worldwide (9). So that *S. Typhi* is highly adaptive in human and it is responsible for persistent as well as life-threatening systemic infection (10). *S. Typhi* may form permanent colonies and biofilm in gallbladder these triggering for establishment chronic asymptomatic carriers state (11). In these carriers state; *Salmonella* resides in gallbladder and it can reach to the organ from an ascending or descending routes .During interaction with their hosts; Microbial pathogens development various strategies for the invasion and adaptation to manipulate environmental signals of host cells (12). DNA damage or perturbation of the cell cycle machinery of the host to create a better environment for bacterial replication is one of these strategies (13). Cytotoxic distending toxin and Colibactin are only two "bacterial genotoxins" (14). Genetic island of *Salmonella* Typhi was discovered in 2004 which contained a homolog to *cdtB*, "gene encoding the active subunit of the Cytotoxic distending toxin" (CDT) (15). CDT is considered an important pathogenesis and can cause cell cycle arrest, cytoplasm distention and apoptosis in a board range of mammalian cells and extend persistence of pathogenic bacteria in the hosts (16). So that Cdt plays important role in the carcinogenic potential of *S. Typhi*; when CdtB reaches to the cytosol, it enters the nucleus of the target cell and induce DNA damage. This is the most acceptable explanation for *S. Typhi* carcinogenicity (17). CdtB activity relies on the expression of 2 genes, pertussis-like toxin A (*pltA*) and pertussis-like toxin B (*pltB*), which can form a CdtB–PltA–PltB tripartite complex that induces DNA damage and cell-cycle arrest, thereby inducing carcinogenesis of gallbladder (18). The aims of this study are screening for *S. Typhi* chronically persisting in gallbladder of patients undergoing cholecystectomy, by using standard culture methods and a highly specific and sensitive PCR assay. We further characterize the isolate for its toxins forming ability by detection of *CdtB*, *PltA* and *PltB* genes by PCR assay .

## 2. MATERIALS AND METHODS

### 1. *Histopathological classification of clinical specimens*

After cholecystectomy is done to gallbladder; it's put in containers which contain Neutral Buffered Formalin 10% for Histopathological classification.

### 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°C overnight (16-20 hours) then Transfer 1 ml of from the inoculated and incubated buffered peptone water with a sterile pipette to 10 ml Tetrathionate broth (Müller-Kauffmann). Incubate at 41.5°C ± 0.5°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°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} (19).

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

Api-20E system is used clinically for the rapid identification of the *Salmonella* Typhi isolates this test done according to Leboffe and Piercr, 2005 (20, 21).

#### 2.4 *Molecular diagnosis of Salmonella Typhi isolates*

##### 2.4.1 *Extraction of genomic DNA from bacterial culture (n=11), DNA extracted from tissue in formalin (n=50) DNA extracted from the formalin-fixe paraffin-embedded gallbladder tissue (n=100)*

Genomic DNA was extracted from *Salmonella* Typhi isolates by using Geneaid Genomic DNA Purification Kit (UK) and done according to company instructions; *Salmonella* Typhi culture has been inoculated in 10 ml LF broth medium and incubated at 37°C overnight in shacking incubator., Genomic DNA was extracted from *Salmonella* Typhi from tissue in formalin (n=50) by using Geneaid Genomic DNA Purification Kit (UK) and 100 of DNA extracted from the formalin-fixe paraffin-embedded gallbladder tissue The extraction from the formalin-fixed paraffin-embedded tissue (n=100) subjected to many process and then Genomic DNA was extracted from *Salmonella* Typhi by using Geneaid Genomic DNA Purification Kit (UK).

#### 2.4 .2 Estimation of DNA Concentration

The extracted genomic DNA is checked by using Nanodrop spectrophotometer which measures DNA concentration (ng/ $\mu$ l) and checks the DNA purity by reading the absorbance at (260 /280 nm) (22).

#### 2.4 .3 Preparation of primers

According to instruction of the primer synthesizer company, the primers (originally lyophilized), were dissolved in the free ddH<sub>2</sub>O to obtain a final concentration of 100  $\mu$ M/ $\mu$ l which served as a stock solution that stored at -20 °C. A concentration of 10  $\mu$ M/ $\mu$ l was prepared from the stock primers to be used as a work primer (23).

#### 2.4 .4 PCR assay for screening of *S. Typhi*

All extracted DNA were screened for three diagnosis genes (*invA*, *viaB* and *H1d*) by a simplex PCR method (8,24) .The composition of the PCR mixture was prepared in total volume 20 $\mu$ l for *invA*, *viaB* and *H1d* genes which done as in table (1).Amplification of these genes were achieved on the Thermo cycler as in table (1,2). The PCR products were visualized by electrophoresis on 1.5% agarose gels in 1X TBE buffer at 95 V for 45 min.

Table (1):- primers used in PCR for detection of *Salmonella Typhi*

Target gene		Sequence (5'-3')	Ta (°C)	Product size (bp)	Accession number
<i>invA</i>	F	5'GTGAAATTATCGCCACGT-TCGGGCAA 3'	66.5	284	M90846
	R	5'TCATCGCACCGTCAAA-GGAACC 3'			
<i>viaB</i>	F	5'GTTATTTTCAGCATAA-GGAG 3'	56	439	D14156.1
	R	5'CTTCATAACCACTTTCCG 3'			
<i>H1d</i>	F	5'ACTCAGGCTTCCCGTAAC-GC-3'	56	763	CP030936.1
	R	5'GGCTAGTATTGTCCTTAT-CGG-3'			

Table (2): PCR condition of *invA*, *viaB* and *H1d* genes

Monople x gene	Temperature ( °C )/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
<i>Inv A</i>	95/5min	94/40 s	66.5/	72/90s	72/10	3
<i>ViaB</i>	94/5min	94/30 s	56/30 s	72/60 s	72/5	3
<i>H1d</i>	94/5min	94/30s	56/30s	72/60s	72/5	3

#### 2.4.5 Screening of *Salmonella Typhi* isolates producing genotoxins

Three genes were selected for Screening of *S. Typhi* producing toxins; these genes are *cdtB*, *pltA* and *pltB*. The composition of the PCR mixture was prepared in total volume 20µl for each gene which done together in Multiplex PCR. The PCR products were analyzed by electrophoresis on 1.2% agarose gels in 19 TAE buffer at 50 V for 85 min. as in table (3,4).

Table (3):- primers used in PCR for detection of *Salmonella Typhi* genotoxins

Target gene		Sequence (5'-3')	Ta (°C)	Product size (bp)	Accession number
<i>cdtB</i>	F	5'GAAACAAGTCAGGCAT-TGCC 3'	56	1059	CP030936.1
	R	5'GAATGGCTCATAAACAC-GCC 3'			
<i>pltA</i>	F	5'GTGGGACTATCATCGTG-CAG 3'	56	1047	CP030936.1
	R	5'AGGGTGATCAACGTAAC-CAC 3'			
<i>pltB</i>	F	5'AGTAGTGAAAACCCA-TCGCG 3'	56	785	CP030936.1
	R				

Table (4): Programs of Multiplex PCR conditions to Toxin genes

Multiple gene	Temperature ( °C )/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
<i>cdtB</i>	94/5 min	94/30 sec	56/30 sec	72/ 1 min	72/ 5 min	3
<i>pltA</i>						5
<i>pltB</i>						

### 3. RESULTS AND DISCUSSION

This study was carried out in high graduated laboratories in the department of biology, college of science / university of Thi-Qar, during the period from December 2019 to October 2021. Gallbladder Undergoing to Cholecystectomy were 200 cases were collected and diagnosed in AL-Hussein teaching, Al-Amel Hospitals and Noor Al- Hussein and Ibn-Al- Baitar laboratories in Thi-Qar Province /Iraq and 50 samples of normal gallbladder from forensic as control during the amount from December 2019 to July 2020 from both sexes and different age. The results of our study are compared with those of well- known authors. After a detailed history and clinical investigations following observations were noted. The results of histological study appeared that there were the most common diseases were Cholecystitis in patient were 79.0 %, and in control were 5.6%. While the less common disease were cancer in patient were 4.2 %, and in control were 0.00%. as in figure (1, 2). While Kareem in 2015 study showed that the incidence of Cholelithiasis was 33(91.66%) of gallbladder

disease (25), this is may be because demographic difference and the different between the number of the sample between these studies.

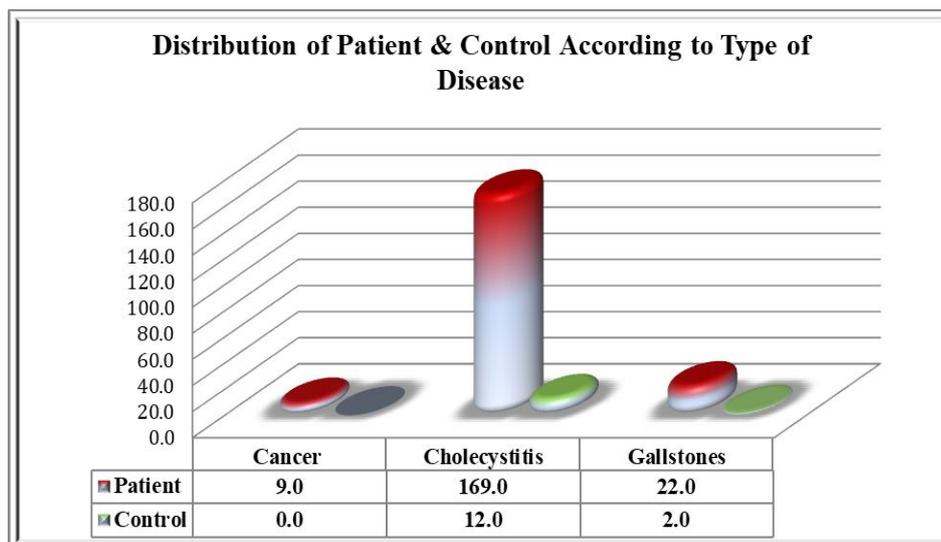


Figure (1): Distribution of patient and control according type of disease

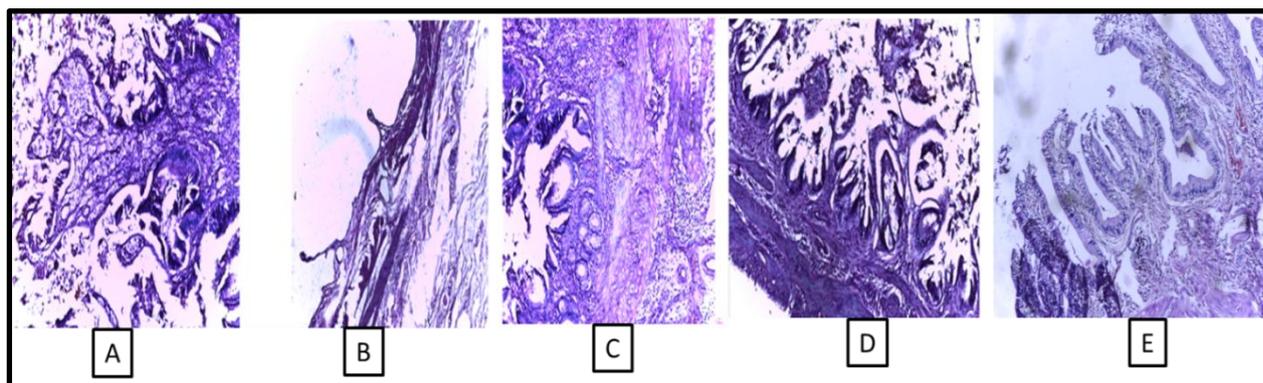
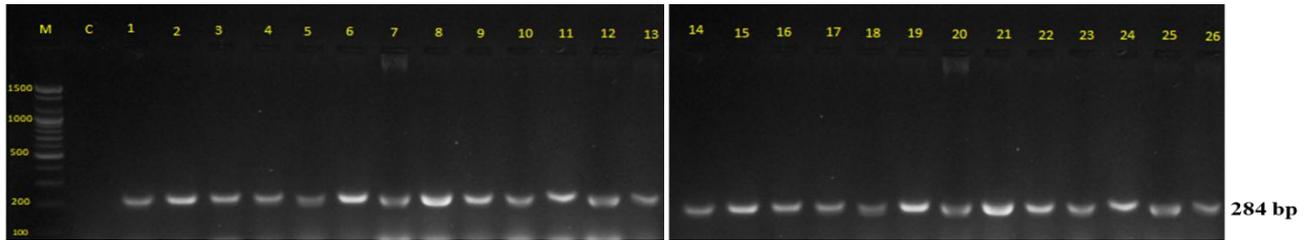


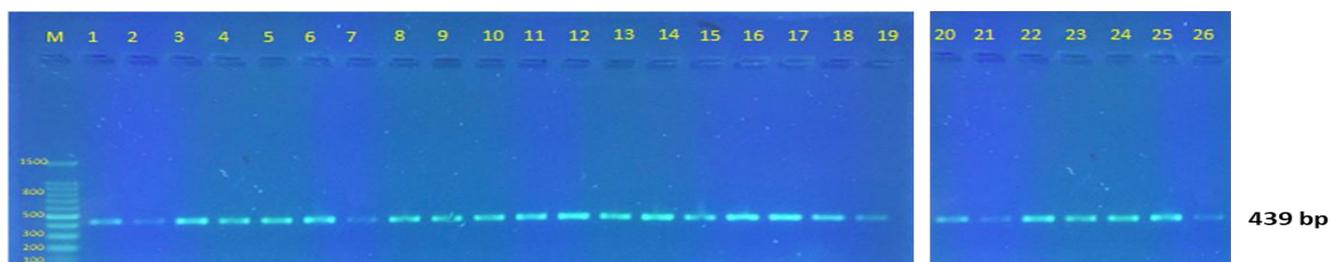
Figure (2): Histological features under a microscope within power 10x showing (A) Gallbladder cancer (b) Gallstones (c) Chronic cholecystitis (D) Acute cholecystitis (E) Normal gallbladder

A total of 11 isolates of *S. Typhi* which identified by conventional biochemical test and API 20- E were subjected to DNA extraction, 50 of DNA extracted from tissue in formalin and 100 of DNA extracted from the formalin-fixe paraffin-embedded gallbladder tissue were subjected to PCR assay for presence of *invA*, *viaB* and *H1d* genes. The prevalence of *Salmonella* Typhi among patients infected with Cancer, Cholecystitis, Galls stones, the result indicate among 200 patient 26 of them infected with *Salmonella* Typhi with percentage (13.06%). A positive results have seen in 26(100%) of sample subjected to PCR assay for presence of *invA*, *viaB* and *H1d* genes as in figure (2,3,4) while Kareem in 2015 found *Salmonella* Typhi only 3(3.5%), of total number of isolates(25), this is may be because demographic difference and the different between the number of the sample between studies. Studies indicate that approximately 90% of the chronic *S. Typhi* carriers have gallstones (26).Nath in 2008 demonstrated that such carriers have 150-fold excess risk of developing hepatobiliary carcinoma and (10-15) fold excess risk of developing gallbladder carcinoma

compared with non-carriers; In addition, chronic carriers have an approximately (8-fold) greater risk of developing gallbladder carcinoma than non-carriers (27).



**Figure(2):**PCR products of the *invA* gene of *Salmonella Typhi*., The size of the PCR product is 284 bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder



**Figure(3):**PCR products of the *viaB* gene of *S. Typhi*.,The size of the PCR product is 439 bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder



**Figure(4):**PCR products of the *H1d* gene of *S. Typhi*.,The size of the PCR product is 763 bp. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder

The studies demonstrated that *S. Typhi* considered one of the pre-disposing factors for gallbladder diseases (28). When it's reaching to gallbladder then establishment an acute, infection accompanied by cholecystitis, or persist in an asymptomatic carrier state to mediate colonization in the gallbladder, this mechanism used by the bacterium in the especially in presence of gallbladder abnormalities such as gallstones (29). However, *S. Typhi* produces bacterial glucuronidase enzyme that leads to precipitation of calcium bilirubinate and leading to formation the gallstone (30). Many studies have been showed that bacteria play an essential role in the formation of brown pigment stones and often the pigment part or nucleus of the mixed cholesterol stones (31). A chronic carrier state in patient with gallstone significantly increases the chance of development a carcinoma of the gallbladder (32). The primary effect of CDT in the target cells is induction of DNA damage; therefore CDT may contribute to the tumorigenic process (33). CDT consists of a heterotrimeric complex of three subunits CdtA, CdtB, and CdtC (34). In many kinds of mammalian cells; this toxin can

induce, arrest the growth and extend persistence (35). CDT has been detected among *Salmonella* serovars especially in *S. Typhi*, and *cdtB* requires two genes *pltA* (pertussis-like toxin A) and *pltB* (pertussis-like toxin B) to induce signs of intoxication eukaryotic cells such as cellular distension and cell cycle arrest (15). The study indicated the all toxin genes were isolated from *S. Typhi* of patients with gallstones, while non *CdtB* isolated from bacteria in patients with Cholecystitis and 84,6% of *S. Typhi* contain *pltA* and *pltB* genes. As in figure (5)

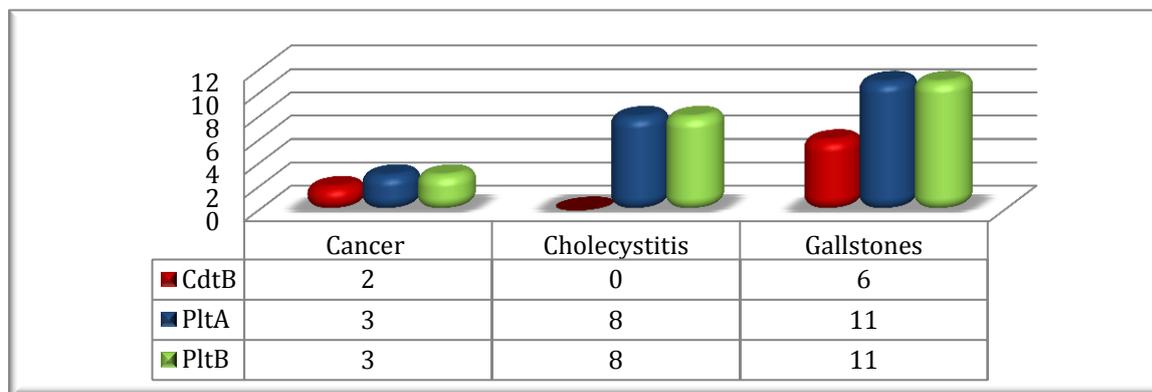
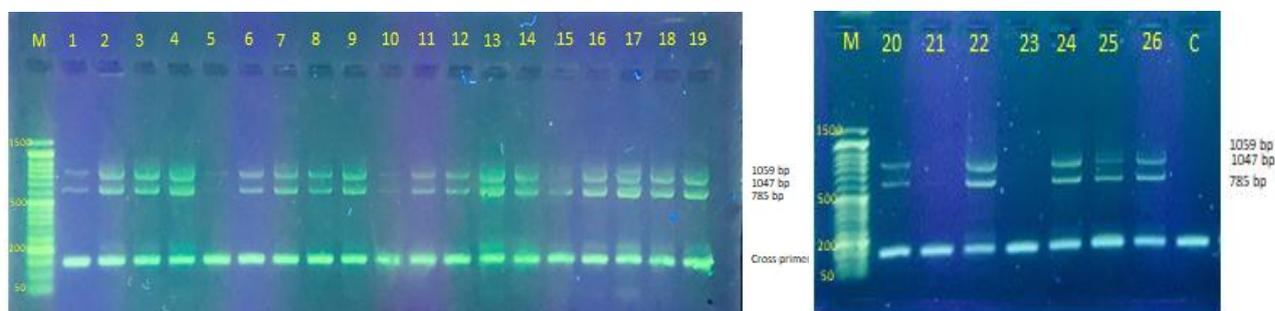


Figure (5)- : Prevalence of toxin genes according to type of disease

The phylogenetic group was determined by using a multiplex PCR method for selected the phylogenetic groups of *S. Typhi* according to PCR detection of the toxin gene, *CdtB*, *PltA* and *PltB* genes. Based on the presence or absence gene marker the results allowed the categorization of isolates into either one of the three major phylo-groups.as in figure (6)



**Figure (6):** PCR products of three *Salmonella Typhi* genes (*cdtB*, *pltA* & *pltB*), the sizes of the PCR products are 1059, 1047 & 785 bp respectively. The gel was 1.5% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder.

Chronic inflammation is an important predisposition factor for developing cancer (36). In multiple cell culture models, administration of purified PltB of *S. Typhi* elicited expression of pro-inflammatory cytokines, possibly suggesting a role of S-CDT in the inflammation induction (37). However, when *S. Typhi* is growing in stranded culture medium; the *cdtB* is not expressed but only expressed when *S. Typhi* reaches to a specific intracellular compartment of host cells (38). It is intriguing that *S. Typhi* is the only *Salmonella* serovar that encodes CdtB. because *S. Typhi* is an exclusively human pathogen; it's tempting to speculate that CDT plays a role in aspects of host-pathogen interactions that are unique to the human host. So that; it's ability to cause long, persistent infections in human are unique *S. Typhi* feature. It is possible that CDT may be involved in some aspects of the establishment of persistent infection, because, at least in other bacteria, this toxin has been shown to possess

immunomodulatory activities (39). More experiments will be required for investigating this hypothesis.

#### 4. CONCLUSION

The most common gallbladder diseases in patient were Cholecystitis (79.0 %); While the less common disease were cancer (4.2 %), The prevalence of *Salmonella* Typhi among patients infected with Cancer, Cholecystitis, Galls stones were (13.06%) and all toxin genes were isolated from *S. Typhi* of patients with gallstones, while non *CdtB* isolated from bacteria in patients with Cholecystitis and 84,6% of *S.Typhi* contain *pltA* and *pltB* genes.

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