Original Article

Isolation and Identification of Bacterial Species from the Human Gallbladders Bile of Sudanese Patients

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ABSTRACT

Background: Gallbladder infections are one of the most important problems that affect Sudanese patients.

Objectives: To isolate bacterial species from infected human gallbladder's bile in Sudanese patients admitted for cholecystectomy due to calcoulus or acalcoulus cholecystitis.

Materials and Methods: A total of 100 bile specimens from 100 patients (88 females and 12 males), were examined in this study. Bile specimens were collected from three different operating theatres including IbnSena Hospital, Sudan Private Clinic and Omdurman Teaching Hospital.

Results: Six bacterial species were recognized in bile specimens, four of them are gram-negative and two are gram- positive species. In the present study, bacteria were isolated from 40 specimens out of the 100 bile specimens cultured with an overall incidence of 40%. It was noted that all positive bacterial bile cultures correlated with the presence of gallstones except three *Salmonellae* which were isolated from bile of acalculus gallbladders. The most prevalent bacteria isolated were *E.coli* which was isolated from 24 specimens out of the 100 bile specimens. On the other hand, *Staphylococcus aureus* and *Pseudomonas* spp. were less frequently isolated from bile specimens showing frequencies of 4 (4%) for each.

Conclusion: The finding of this study indicated that *Escherichia coli* were the most prevalent bacteria which isolated from human bile. As well as, the study revealed that certain bacterial species such as Salmonellae possess characters which allow them to cause cholecystitis without need to gallstones formation.

Key wards: Gallbladder Bile, Bacterial isolates, Bile specimens, Cholecystectomy; Bacterial cholecystitis, Acalculus gallbladders.

Acute cholecystitis is the inflammation of the gallbladder wall, and is one of the most common complications of symptomatic gallstones, which requires surgical intervention[1]. It is due to impaction of gallstones in the cystic duct or neck of the gallbladder, then the gallbladder becomes distended and inflamed[2].

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Acalculous cholecystitis is more common in men, in this setting calculi obstructing the cystic duct are not found. Parasitic infestation and primary gallbladder infection by *Salmonella typhi* represent risk factors for acalculous cholecystitis[3]. Chronic cholecystitis may denote exclusively the presence of chronic inflammation or cholelithiasis. It can be primary or secondary chronic cholecystitis. The common symptoms of chronic cholecystitis are biliary colic and a steady pain located in the epigastrium or right upper quadrant of the abdomen[4]. Cholecystectomy (removal of the gallbladder) is either performed by the open method or by using laparoscopic

technique. It is still the most efficient method for treatment of gallbladder diseases. Open cholecystectomy requires an incision in the anterior abdominal wall of 12 to 20 cm in length[5]. Laparoscopic cholecystectomy is usually performed with special attention. A laparoscope is inserted through several small incisions in the abdomen; the gallbladder is then withdrawn through one of the incisions[2]. Bacterial infections of the human gallbladder may play a role in about 50% of patients early in the course of acute cholecystitis, and within a few days, bacteria can be cultured from the bile of nearly all patients[3]. In those patients the microorganisms most frequently isolated by culturing of gallbladder bile included *E. coli*, *Klebsiella* species, Group D *Streptococci*, *Staphylococcus* species, *Pseudomonas aeruginosa*, *Proteus* species, *Clostridium perfrengens*, and *Bacteroides*species[6].

Many studies in different parts of the world have been performed to illustrate the bacteriology of the gallbladder bile and bacteria that commonly isolated from bile in all disease states of the biliary tract were primarily Gram negative Enterobacteria, in which *Escherichia coli* alone accounting for more than 50% of the cases; others, such as *Klebsiella*, *Enterobacter* and *Proteus* were less common. *Enterococcus faecalis* was also common; anaerobes such as *Clostridium perfringens* were rare[7, 8, 9, 10, 11]. Bacterial cholecystitis, particularly in female subjects, is increasing nowadays. Epidemiological data for bacterial infections of the gallbladder in the Sudanese population is still lacking. The purpose of this study is therefore, to throw light on the prevalence of bacterial infections of the gallbladder in the Sudanese patients, and to identify the bacterial species in the gallbladder bile of them using a variety of approaches and numbers of techniques.

MATERIALS AND METHODS:

Ethical consideration: An ethical clearance was obtained from the authorities of Ibn Sina Hospital, Omdurman Teaching Hospital and Sudan Private Clinic. Informed consents were obtained from the studied patients before interviewing and the aims of the study were explained clearly to them.

Study design: The research was performed as a descriptive survey study.

Study area: Bile samples used in the study were provided from three different operating theaters belonging to Ibn Sina Hospital, Omdurman Teaching Hospital and Sudan Private Clinic, Khartoum, Sudan.The research experiments were conducted during January 2003 to July 2003 and the laboratory work was carried out in the Central Veterinary Researches Laboratory, Soba- Khartoum- Sudan.

Study population: Inclusion criteria for patient's selection: all patients diagnosed as having a calculous or acalculous cholecystitis and admitted for cholecystectomy either by open or laparoscopic method who agreed to be included in the study.

Data collection: The demographic data including some variables such as gender, age, residence type of operation and post-operative data such as gall stones size, shape and type were collected by closed ended questions questionnaire. The size of each stone was then estimated by measuring its diameter and expressing that in mms. The gallstones were examined afterwards to detect their shape and form; they are described as irregular, round or faceted gallstones. The type and chemical composition of the gallstones were then delineated, depending on ultrasound reports and visual observation; they are designated as cholesterol, pigmented or mixed type of gallstone.

Collection and transport of bile specimens: 100 aspirated bile samples

were obtained from removed gallbladders of patients (88 from females and 12 from males) undergoing elective open and laparoscopic cholecystectomy. The specimens were collected aseptically using disposable 5 cc syringes at the time of surgery. In three occasions the gallbladders were fibrotic and there was a little amount of bile, swabs were used in such cases to take bile specimens from those gallbladders and then they were streaked on the transport media. All specimens were transported to the laboratory immediately by using Stuart transport media.

Primary culturing (aerobic and anaerobic): Four types of culture media were used for primary culturing of bile specimens. Choice of these culture media depend upon their ability to allow the growth of the organisms suspected to be present in the bile specimens. The culture media used include macConkey agar, blood agar medium, nutrient agar and selenite broth.

Techniques for culturing of bile specimens: The surface of the agar medium prepared in Petri dishes can be inoculated with specimens by several methods[18]. In this study we used the standard method for culturing the bile specimens.

Streaking methods for isolating single bacterial colonies from bile: Once the primary inoculum from bile specimens was made, a wire loop was use to spread the material into the four quadrants of the plate, as described by Koneman *et al.*[12]. The wire loop was sterilized between each successive quadrant streak. The purpose of this technique is to dilute the inoculum on the surface of the agar medium, so that single isolated colonies of bacteria, known as colony forming units (CFU) can be isolated.

Conditions of incubation and interpretation of cultures results: All the examined bile samples which were cultured on macConkey agar, blood agar and selenite broth medium were incubated aerobically and anaerobically at 37 °C for 24 hours. The identification methods of bacteria used were dependent upon changes in culture media after bacterial growth. This aided in choosing further specific set of biochemical tests for identifying the bacterial species.

Biochemical tests: We followed the methods of Barrow and Feltham[13] as described in the first and second stage tables for bacterial identification. The followings biochemical tests were used; oxidase test, indole test, methyl red test and voges proskauer test, catalase test, coagulase test, urease test, citrate utilization test, motility test and carbohydrates fermentation test.

Statistical analysis: Data were analyzed by using the statistical package for social sciences (SPSS-10.0). Chi- Square was used when appropriate, and P-value of less than 0.05 was considered significant.

RESULTS:

In the present survey, a total of 100 patients who underwent cholecystectomy (88 females and 12 males) and their ages ranged from 23 to 86 years with a mean age of 46.22 years (±SD 12.399) were investigated for the presence of bacteria in their gallbladders bile. All patients were Sudanese belonging to different socio-economic classes. Table 1 shows the frequency of different bacterial species isolated from bile specimens examined. Sixty bile specimens (60%) out of the 100 specimens examined showed no aerobic or anaerobic bacterial growth. Forty specimens only (40%) showed marked aerobic bacterial growth and bacteria present were isolated and identified thereafter, *Escherichia coli* was the most prevalent bacterial species and was isolated from 24 bile specimens (24%).

Table 1: Frequencies and percentages of bacteria isolated from positive gallbladder's bile cultures.

Bacteria isolated	Frequency	percentage %
No growth	60	60
Escherichia coli	24	24
Staphylococcus aureus	4	4
Pseudomonas spp.	4	4
Salmonella spp.	3	3
Listeria monocytogenes	3	3
Citrobacter freundii	2	2
Total	100	100

Other species including *Staphylococcus aureus* and *Pseudomonas* spp. were less frequently isolated from bile specimens showing frequencies of 4 (4%) for each (Table 1). *Salmonella* spp. were isolated from 3 bile specimens, *Listeria monocytogenes* isolated from 3 bile specimens and *Citrobacter freundii* from 2 bile specimens with percentages of 3%, 3% and 2% , respectively. Bile specimens containing more than one bacterial species were not recorded in this study, only one bacterial species was isolated from each specimen. All the *Staphylococcus aureus* and *Citrobacter freundii* strains isolates were recovered from bile of gallbladders removed by open cholecystectomy. *Escherichia coli* strains were predominantly isolated from bile obtained from gallbladders removed by open cholecystectomy. *Pseudomonas* spp., *Salmonella* spp.and *Listeria monocytogenes* were also most frequently isolated from bile of gallbladders removed by open cholecystectomy.

Bacteria were isolated from 40 out of the 100 bile specimens examined with an overall incidence of 40%. Bacteria were

Table 2: Number and percentages of positive bile cultures obtained from gallbladders removed by open and laparoscopic cholecystectomy.

Type of Surgical operation	No. of gallbladders removed	No. of gallbladders with positive bacterial cultures	%of positive Gallbladders
Open cholecystectomy	79	33	41.77
Laparoscopic cholecystectomy	21	7	33.33

$\chi^2 = 2.652$ $P = 0.851$

Table 3: Relation of positive bacterial bile cultures to different diameters of gallstones.

Bacterial species isolated from gallbladder's bile	Stone diameter / mms				Total
	1-15	16-25	26-45	Acalculous cholecysitis	
No growth	45	11	4	-	60
Staphylococcus aureus	3	1	-	-	4
Salmonella spp.	Nil	Nil	Nil	3	3
Escherichia coli	23	1	-	-	24
Pseudomonas spp.	2	1	1	-	4
Citrobacter freundii	2	-	-	-	2
Listeria monocytogenes	2	1	-	-	3
Total Number of gallbladders	77	15	5	3	100
Total bacterial isolates	32	4	1	3	40

$\chi^2 = 0.0536$ $P = 0.8169$

presented in the gallbladder's bile of 33 patients out of 79 patients who underwent open cholecystectomy (41.77%) while only 7 from 21 patients whom their gallbladders were removed using laparoscopic procedures showed positive bile cultures (33.33%). The results showed that bacteria were more prevalent in bile specimens obtained from gallbladders removed by open cholecystectomy compared to laparoscopic cholecystectomy, but the difference was not statistically significant (P > 0.05). However, from the 40 positive bile specimens 33(82.50%) were obtained by open cholecystectomy, while only 7(17.50%) were obtained by laparoscopic cholecystectomy (Table 2).

Table 4: Relation of positive bacterial bile cultures to number of gallstones in the gallbladder.

Bacterial species isolated from gallbladder's bile	Gall stones numbers				Total
	1-15	16-25	>25	Acalculous gallbladder	
No growth	53	4	3	-	60
Staphylococcus aureus	2	-	2	-	4
Salmonella spp.	Nil	Nil	Nil	3	3
Escherichia coli	20	2	2	-	24
Pseudomonas spp.	3	-	1	-	4
Citrobacter freundii	2	-	-	-	2
Listeria monocytogenes	3	-	-	-	3
Total number of gallbladders	83	6	8	3	100
Total bacterial isolates	30	2	5	3	40

$\chi^2 = 0.0526$ $P = 0.8187$

Table 5: Relation of positive bacterial bile cultures to the shape of gallstones in the gallbladder.

Bacterial species isolated from gallbladder's bile	Gallstones shape				Total
	Irregular	Round	Faceted	Acalculous gallbladder	
No growth	25	28	7	-	60
Staphylococcus aureus	4	-	-	-	4
Salmonella spp.	Nil	Nil	Nil	3	3
Escherichia coli	23	1	-	-	24
Pseudomonas spp.	3	1	-	-	4
Citrobacter freundii	2	-	-	-	2
Listeria monocytogenes	1	2	-	-	3
Total No. of gallbladders	58	32	7	3	100
Total bacterial isolates	33	4	Nil	3	40

$\chi^2 = 0.1045$ $P = 0.003$ Spearman correlation test $P = 0.0001$

The majority of gallbladders examined in the study (97 specimens) contained gallstones (97% from total number of specimens), whereas 3 gallbladders only were acalculous (3%). Regarding the size, gallstones were categorized into 3 groups having diameters of 1-15 mms, 16-25 mms and 26-45 mms (Table 3). Most gallbladders examined (77%) had gallstones of diameter size 1-15mms. Large gallstones of diameters 16-25 mms and 26-45mms were detected in 15% and 5%, respectively of the gallbladders examined. Most gallbladders studied (86% from the total number of gallbladders)

contained 1-15 gallstones. Gallbladders containing 16-25 gallstones or containing more than 25 gallstones were less, representing 6% and 8%, respectively from the total number of gallbladders examined. A gallstone in this study exhibits an irregular, rounded or faceted shape. The irregular shape was predominant in this study. 58% of the total gallbladders examined carried irregularly shaped gallstones. The round and faceted shaped gallstones were contained in 32% and 7% of the total gallbladders examined (Table 5). Pigmented, cholesterol and mixed types of gallstones were all found in gallbladders examined. Gallstones composing of cholesterol were more encountered in this study and were detected in 39 gallbladders (39% of the total gallbladders studied). The mixed and pigmented gallstones were less frequent, encountered as 36% and 22% respectively of the total gallbladders examined (Table 6).

Bacterial isolation frequencies were higher 80% (32 bacterial isolates) in bile specimens obtained from gallbladders with gallstones diameter in the range of 1-15 mms. Gallbladders with gallstones diameter ranges of 16-25 mms and 26-45 mms had lower frequencies of bacterial isolates from bile showing 10% (4 bacterial isolates) and 2.5% (one bacterial isolate), respectively (Table 3). The result indicates that gallstones with diameters of 1-15 mms facilitate bacterial growth and multiplication in bile. However, this finding was not statistically significant (P>0.05). Gallstone sizes revealed no positive association with bacterial isolation from bile. Strikingly, 3 *Salmonella* spp. were isolated from 3 bile specimens obtained from acalcubus gallbladders. The result may show that bile of acalculous gallbladder is inhibitory to the growth of other bacterial species but not to *Salmonella* spp. *Escherichiacoli* was the predominant bacterial species isolated from bile in the present study. It was isolated from 24 bile specimens. Interestingly, 23 of those strains were isolated from bile specimens of gallbladders containing gallstones of 1-15 mms in diameter (Table 3). Bacteria were more prevalent 75% (30 isolates) in bile specimens obtained from gallbladders containing 1-15 gallstones. Bacteria were less frequently isolated from bile of gallbladders containing 16-25 gallstones or more than 25 gallstones, showing 2 and 5 bacterial isolates, respectively (Table 4).

Table 6: Relation of positive bacterial bile cultures to the type of gallstones in the gallbladder

Bacterial species isolated from gallbladder bile	Gallstone type				Total
	mixed	cholesterol	pigmented	Acalculous gallbladder	
No growth	12	35	13	-	60
staphylococcus aureus	3	-	1	-	4
Salmonella spp.	Nil	Nil	Nil	3	3
Escherichia coli	18	1	5	-	24
Pseudomonas spp.	2	1	1	-	4
Citrobacter freundii	-	-	2	-	2
Listeria monocytogenes	1	2	-	-	3
Total number of gallbladders	36	39	22	3	100
Total bacteria Isolates	24	4	9	3	40

$\chi^2 = 12.804$ $P = 0.0004$ Pigmented vs. cholesterol; Mixed vs. cholesterol and pigmented

Statistically, those variations in bacterial isolates from bile of gallbladders containing different numbers of gallstones were not significant (P>0.05). Number of gallstones contained in the gallbladder has no positive association with the isolation

of bacteria from bile. Positive bacterial bile cultures were more prevalent in bile of gallbladders containing irregularly shaped gallstones 82.5% (33 bacterial isolates). No bacterial growth was detected in bile specimens obtained from gallbladders containing faceted gallstones while only 10% (4 bacterial cultures) were positive for bile obtained from gallbladders carrying round shape gallstones. Those variations in positive bacterial bile cultures observed in bile obtained from gallbladders containing different shapes of gallstones were statistically significant ($P<0.05$). There is significant statistical number of bacterial isolates in bile of gallbladders containing irregularly shaped gallstones compared to bile of gallbladders containing rounded or faceted shape gallstones (Table 5). Bacterial isolates were more prevalent in bile obtained from gallbladders containing mixed types of gallstones 60% (24 bacterial isolates). Less bacterial isolates were recovered from bile of gallbladders carrying either pigmented 22.5% (9 bacterial isolates) or cholesterol 10% (4 bacterial isolates) types of gallstones (Table 6). However, there was significant bacterial isolates in bile with pigmented gallstones compared to bile with cholesterol gallstones ($P<0.05$). Moreover, there was significant numbers of bacterial species isolated from bile with mixed type of gallstones as compared to cholesterol and pigmented types of gallstones ($P< 0.05$). This result data shows a strong relationship between isolation of bacteria from bile and the type of gallstones carried in the gallbladder (Table 6).

DISCUSSION:

In the present study most of the patients admitted for cholecystectomy were females. From 100 bile specimens examined 88 belonged to females and 12 belonged to males. Predominance of females was also observed in the study of Al-Harbi *et al.*[11] in Saudi patients and Ibn Ouf *et al.*[14] In Sudanese patients. Gallstones disease, which requires cholecystectomy, has different distribution over the world. The incidence and frequencies of gallstones disease occurring in developed countries are high[3]. In the United States the incidence has a range of 30% to 70% while in other developed countries such as Western European countries and Japan those percentages decreased, showing a range of 10% to 30% from the total population of each country. In under developed countries such as East and West Africa and in Egypt the incidence of gallstones disease is very rare; it has a range of 0 to 1% from the total population of each country[3]. It seems that the sophisticated way of life and types of food play a role in this higher incidence of gallstones disease in developed countries. The prevalence of gallstones disease increases dramatically in females, reaching up to 70% in women of 30 years old[4]. This finding coincides with our results in the present study as 85% of women of varying ages examined showed an incidence of gallstones disease. In this study bacterial prevalence in bile specimens examined was 40%. This incidence was similar to that reported by Csendes *et al.*[7] in Chile, Ohdan *et al.*[8] in Japan and Darko and Archampong[9] in Ghana. However, the incidence of bacteria in bile reported in our study was higher than that reported by Al-Harbi *et al.*[11] in Saudi Arabia (25%) and lower than that reported by Keighly *et al.*[15] in United Kingdom (53%). This indicates that the incidence of bacteria in bile is variable in different countries and regions of the world[1, 11]. Bile specimens obtained from gallbladders removed by open cholecystectomy revealed more bacteria than gallbladders removed by laparoscopic cholecystectomy. This finding agrees with that of El-Jawadi *et*

al.[16]. However, in our study no significant statistical association was observed between the type of cholecystectomy performed and the number of bile specimens showing positive bacterial cultures.

In this study, the most prevalent bacterial species isolated from bile was *Escherichia coli*. Similarly, *Escherichia coli* were also predominantly reported in bile cultures in surveys performed by Darko and Archampong [9]. In our study *Escherichia coli* was isolated from 24 out of the 40 positive bile specimens. Petakovic *et al.*[17] also cultured *Escherichia coli* from 32 bile specimens out of a total of 240 bile specimens. However, *Escherichia coli* were isolated in lower rates in the study of Darko and Archampong[9], Al- Harbi *et al.*[11] and Chang *et al.*[18]. In the present survey, other organisms were much less frequently isolated which is in agreement with the findings of Darko and Archampong[9], Al-Harbi *et al.*[11] Petakovic *et al.*[17] and Roa *et al.*[19], *Pseudomonas* spp. and *Staphylococcus aureus* each was isolated from 4 bile specimens, *Salmonella* spp. and *Listeria monocytogenes* each was isolated from 3 bile specimens, while *Citrobacter freundii* was isolated from 2 bile specimens only. *Pseudomonas* spp.and *Staphylococcus aureus* were also isolated in the studies of Darko and Archampong[9], Al-Harbi *et al.*[11], Petakovic *et al.*[17] and Chang *et al.*[18]. In our study the 3 *Salmonellae* isolated were not serotyped. Roa *et al.*[19] isolated *Salmonella* spp.from bile in a survey study performed at Chile. Darko and Archampong[9] also isolated 7 *Salmonella* spp.from bile in a survey preformed in Ghanaians patients. Lo *et al.*[20] in Taiwan isolated *Salmonella* group-D from a patient presented with acalculous cholecystitis. *Listeria monocytogenes* and *Citrobacter freundii* are less commonly encountered in bile. *Citrobacter freundii* was isolated from a few bile specimens by Roa *et al.*[19] and by Petakovic *et al.*[17]. *Listeria monocytogenes* appears to be isolated for the first time from bile in this study. It is primarily an animal pathogen[13] and infects man mostly through ingestion of contaminated food[21]. *Escherichia coli* have been incriminated in infectious diseases involving virtually every human tissue and organ[12]. It is the common organism causing urinary tract infection (UTI) and gallbladder infection[22,23,24]. Species of *Escherichia coli* causing UTI belong to the normal microflora of the colon and feces[12]. These organisms are described to have characteristic pili which allow their attachment to the uroepithelial cells[21]. This attachment is necessary for the organisms to start their infectious process. It is not known in the present study whether those strains of *Escherichia coli* isolated from bile belong to the endogenous microflora of the colon and feces or they came from an exogenous source. It is also not defined whether they have certain pili characteristics which allow their attachment to the epithelial surface of the gallbladder.

Bile is known to be inhibitory to bacterial growth and multiplication for its contents of bile salts[13,25]. However, certain bacterial pathogens like *Salmonella* species are able to grow and multiply in it[13]. All bacterial species and strains isolated in the present study were isolated from bile of calculous gallbladders, except the 3 *Salmonella* spp. which were isolated from acalculous gallbladders. This finding shows that the presence of gallstones may change the chemical characteristics of bile so that it allows the growth and multiplication of bacteria[7]. In this study only one bacterial species was isolated from each bile specimen. This indicates that aseptic measures employed during collecting and processing of specimens was adequate. On the contrary, Al-Harbi *et al.*[11] isolated more than one bacterial species from one

bile specimen, which increases the possibility of external bacterial contamination of the specimen[12]. No anaerobic bacterial species were isolated in our study, which coincides with the findings of Al- Harbi *et al.*[11] and Sianesi and Berri[26]. Nielsen and Justesen[27] however, were able to isolate anaerobic bacteria from gallbladder's bile collected from patients presented with a variety of biliary tract diseases. Anaerobic bacteria were also isolated from bile specimens of a group of patients having gallbladders stones [7] whereas, patients with biliary tract or common duct obstruction not due to stones did not yield bacteria on culture.

CONCLUSIONS:

It seems that prevention of bacterial cholecystitis can be achieved by avoiding formation of gallstones, which correlate significantly with the presence of bacteria in bile. However, bacteria may grow and multiply in gallbladder's bile without development of gallstones. In the present study we isolated three *Salmonella* species from acalculous gallbladders. This finding may show that certain bacterial species possess characters which allow them to cause cholecystitis without need to gallstones formation. Our study did not show precisely how bacteria can gain access into the gallbladder. It is obvious that those organisms may originate from an endogenous or an exogenous source, but their presence in the gallbladder protects them from the action of antibodies as well as antibiotics. The incidence of bacteria in the gallbladder bile was 40 % reported in the present study which is similar to that reported in undeveloped countries, while the higher incidence of bacteria in females compared to males needs further investigation.

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