

# Detection of bacterial DNA in cholesterol gall stones

N Khare, D Sharma, U Somashekar, A Prakash, S Prakash, M Mendki, A Anvikar

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## Abstract

Introduction: Recently, bacterial infection has been shown to play an important role in the formation of cholesterol gallbladder stones. This led us to investigate the presence of bacterial structures in cholesterol gallstones by means of bacterial culture and DNA detection.

Method: Gallstones were obtained from 31 consecutive patients operated from May 2005 to October 2006 in the department. Culture study was done using standard bacteriological methods. Cholesterol was estimated by spectrophotometry and stones were divided into cholesterol stones (>70% cholesterol) and mixed/pigment stones (<70% cholesterol). DNA for organisms was extracted from the core of the stone and confirmed by agarose gel electrophoresis. Sterile stones were subjected to PCR amplification.

Results: Age of patients ranged from 27 to 66 years (mean = 46.58 years); 14/31 stones were pure cholesterol stones (45%), and 17 (55%) were mixed and pigment stones. On culture, 12 stones (39%) were sterile, while 19 (61%) showed presence of viable bacteria; amongst cholesterol stones, 11 out of 14 were sterile (79%), while only 1 (6%) out of 17 pigment and mixed stone was sterile. E.coli was the most common bacterium isolated (42%) followed by Pseudomonas and Salmonella. DNA extraction was positive in all 31 of the stone samples collected (100%). PCR amplification was positive in all the bacteriologically sterile stones.

Discussion: All gallstones, including cholesterol stones, revealed bacterial DNA; suggesting that bacterial infection is associated even with cholesterol gallstones.

## INTRODUCTION

Cholesterol super-saturation in bile is necessary for cholesterol gallstones to form, but not all people with supersaturated bile form gallstones. Obviously, other overt mechanisms are required for ultimate formation of gallstones.[1] Recently, arrival of molecular genetic evidence of bacterial colonization of cholesterol gallstones compelled researchers to think of an association between the bacterial infectious process and the resultant cholesterol gallstones. It prompted us to investigate the presence of bacterial structures in cholesterol gallstones by means of bacterial culture and DNA detection.

## METHOD

This study was carried out from May 2005 to October 2006 in the Department of Surgery, NSCB Government Medical College, Jabalpur (MP). Culture analysis was done in the

Regional Medical Research Centre for Tribals, ICMR Jabalpur (MP) India. DNA extraction and PCR amplification was done in the Defense Research and Development Establishment, Entomology Division, Gwalior (MP) India.

## SAMPLE COLLECTION

The study was performed on gallstones obtained from 31 consecutive symptomatic patients who underwent open cholecystectomy in the department of surgery. In all the cases, the cause of symptoms was confirmed by ultrasonography. Twenty-eight patients were operated for chronic calculous cholecystitis, 3 patients were diagnosed as empyema of the gallbladder. A single shot of Ceftriaxone was administered at the time of induction of anesthesia; empyema patients received antibiotics for 5 days post-operatively.

Gallstones were collected intra-operatively by cutting open

the removed gallbladder by sterile instruments (on a separate trolley). All stones were collected in a sterile container and sealed.

### CULTURE STUDY

Stones were cut at the mid point after sterilizing the outer surface by washing it in alcohol and saline. All the stones were hard; none of the stones was amorphous. Sample for culture analysis was taken from the core of the stone and not the shell, so as to avoid culture getting contaminated with bile or gallbladder infection. Gallbladder tissue, bile and blood were not cultured.

100mg gallstone sample was inoculated in nutrient broth medium (Himedia grade) and incubated in a Biochemical Oxygen Demand (BOD) incubator at 30°C for 48 hours. The contents were plated into nutrient agar petriplates and reincubated in the BOD incubator at 30°C for 48 hours. Thereafter, colony characteristics, colony smear and bacterial motility tests were analyzed. Biochemical tests for bacterial identification were done by Indole, Methyl Red, Voges Proskauer and Catalase (IMViC) following which Triple Sugar Iron (TSI) agar slant reactions were performed.[2]

### CHOLESTEROL ESTIMATION

Cholesterol estimation was done to differentiate cholesterol stones from mixed stones. 100mg gallstone was dried at 105°C for 3 hours. The stones were then dissolved in lysis buffer and centrifuged at 2000rpm for 10 minutes. The pellets were discarded and the supernatant was collected. Cholesterol was estimated by spectrophotometry at 722nm and was calculated as percentage of dry weight of stone. Stones were divided into cholesterol stones (>70% cholesterol) and mixed and pigment stones (<70% cholesterol).[3]

### DNA EXTRACTION

100mg gallstone sample was inoculated in nutrient broth medium (Himedia grade) and incubated in the BOD incubator for 48 hours at 30°C. The mixture was centrifuged at 2000rpm for 10 minutes. The supernatant was discarded and the pellets were collected and washed with phosphate buffer saline and sterile distilled water. The pellets were then added to Tris-HCL Acetic acid EDTA (TAE) buffer (pH 8.0) and cell lysis was carried out by rapid heating at 70°C and cooling at 4°C. The contents were centrifuged at 10000rpm for 10 minutes and the supernatant was collected as template DNA. DNA extraction was confirmed by agarose gel

electrophoresis. 1% Agarose was prepared in TAE buffer and 3µl template DNA was loaded. The mixture was run at 100 Volts for 30 minutes and the products were visualized under UV light at 312nm (UVItech, UK).[3]

### PCR AMPLIFICATION

The PCR reaction mixture was prepared by adding 1µl 10X PCR Buffer, 25mM MgCl<sub>2</sub>, 2mM dNTP Mixture, 16F27 forward primer (5' CCA GAG TTT GAT CMT GGC TCA G 3') and 16R1525XP reverse primer (5' TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC 3')[4]; 0.5µl each of distilled water and Taq DNA polymerase (5U/ul) were added; 3.0µl of DNA was added for amplification. Initial denaturation was done at 94°C for 5 minutes. Further, denaturation was done at 94°C for 1 minute, annealing was done at 55°C for 1 minute and extension was done at 72°C for 1 minute. The reaction mixture underwent final extension at 72°C for 10 minutes and was cooled at 4°C; 35 such cycles were run in a 200µl thin-walled PCR tube with an i-cycler thermal cycler (Biorad Ltd.) and the products were visualized under UV at 312nm (UVItech, UK).

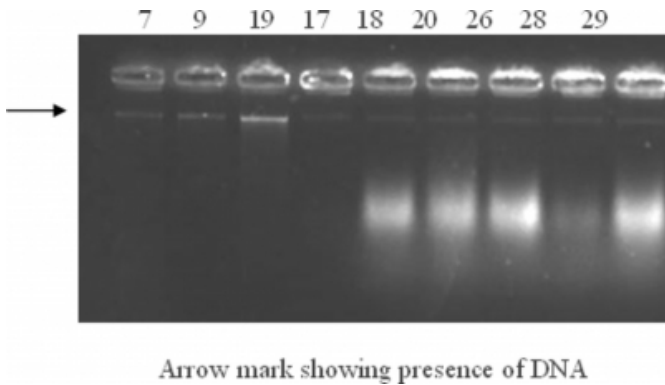
### RESULTS

Age of patients ranged from 27 to 66 years (mean 46.58 years). Fourteen out of 31 stones analyzed were pure cholesterol stones (45%), while 17 (55%) were mixed and pigment stones. Twelve stones (39%) were sterile on culture, while 19 (61%) showed presence of viable bacteria. Amongst the cholesterol stones, 11 out of 14 were sterile (79%), while only 1 (6%) out of 17 pigment and mixed stone was sterile on culture. E.coli was the most common bacterium isolated (n = 8/19, 42.1%) followed by Pseudomonas (n = 2/19, 10.5%), Salmonella (n = 2/19, 10.5%), Klebsiella (n = 1/19, 5.2%) and S. aureus (n = 1/19, 5.2%).

DNA extraction was successful in all 19 culture positive stone samples (Figure I).

Figure 1

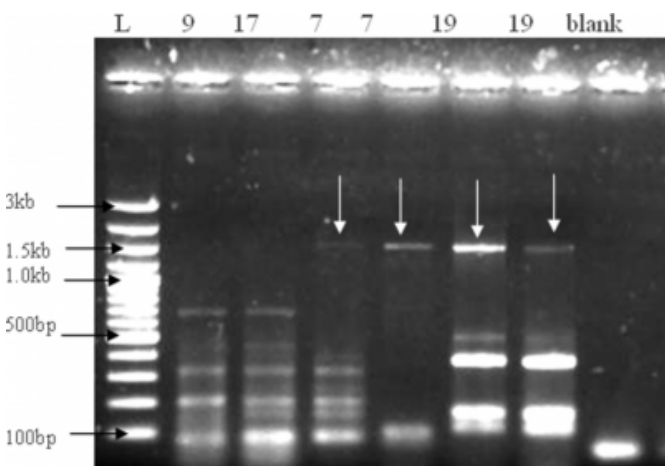
Figure 1: DNA extracted from the core of the gallstone



Sterile stones were subjected to PCR amplification and 16S rRNA PCR amplification showed presence of bacteria in all the sterile gallstones (Figure II).

Figure 2

Figure 2: 16S rRNA PCR amplification showing presence of bacteria in gallstone



## DISCUSSION

Though the relative frequencies of different bacteria are variable, probably owing to the geographic variations, *E. coli* remains the most common organism isolated in almost all the studies including the present one. (Table I).<sup>[3, 5,6,7]</sup> In our study, a high percentage of cases had mixed infection. The majority of cholesterol stones in the present study were reported to be sterile (78.6%), while 94.1% of pigment stones were positive on culture study. Similar results have been reported in other studies. Any difference in clinical presentation, vis-à-vis different type of bacterial infection, was not seen in the present study. However, a recent study has shown that bacteria sequestered in cholesterol stones cause less infectious manifestations than bacteria in pigment stones.<sup>[8]</sup> Of late, many animal and clinical studies have

demonstrated the presence of *H. pylori* in gallstones, but their real significance remains to be elucidated.<sup>[6, 9]</sup>

A recent Japanese study detected bacterial DNA in 26/30 brown pigment stones (87%), in 12/21 pure cholesterol stones (57%) and in 12/18 mixed cholesterol stones (67%). Eighty percent of bacteria in brown pigment stones were gram-negative rods or anaerobes. In contrast, quite surprisingly, 100% of bacteria in pure cholesterol stones were gram-positive cocci. The bacteria in mixed cholesterol stones consisted of 40% gram-positive cocci, 50% gram-negative rods, and 10% anaerobes. They concluded that gram-positive cocci are associated with formation of pure cholesterol gallstones.<sup>[10]</sup>

Wu and colleagues from China found bacterial isolates in 26/30 cholesterol gallstones.<sup>[3]</sup> One of the earlier German studies found that most cholesterol gallstones harbor bacterial DNA (16 of 17 patients), but no bacterial DNA was found in the gallstones with cholesterol content of >90% (3 patients).<sup>[11]</sup> Many researchers have used a culture-independent, molecular genetic approach to detect bacterial DNA in culture-negative cholesterol stones.<sup>[12, 13]</sup>

It was earlier hypothesized that bacterial species may augment the process of nucleation and gallstone growth by contributing specific enzyme activities or secretion of biofilm; resulting in the formation of insoluble precipitates in bile, or by acting as a nidus upon which the deposition of cholesterol crystals may initiate gallstone formation.<sup>[14, 15]</sup> Recently, it has been shown that different bacterial factors facilitate gallstone formation of different types.<sup>[16]</sup>

In our study, DNA was extracted from 100% of the sample. The DNA extraction rate was not affected by the chemical composition of the stone; as cholesterol as well as pigment stones showed presence of bacterial DNA. Even in those stones which were sterile on culture analysis (revealing no viable bacteria), bacterial DNA was confirmed by agarose gel electrophoresis and PCR amplification. It has been postulated that DNA patterns change with organism, but we detected only the presence of DNA, details of species-specific DNA patterns were not studied due to lack of specific primers.

It is possible that some degree of infection provides the trigger required for the stones to form. This could be the answer to the question of cholesterol super-saturation of bile not uniformly translating into cholesterol stones. We did not attempt to identify the specific species and hence PCR

amplification, using universal bacterial primer, was done only on the culture-negative stones. Detection of DNA, in cholesterol stones adds to the growing pool of studies finding an infectious nidus in these gall stones.

## **CONCLUSION**

Our data shows presence of bacterial DNA and thus the association of bacterial infection with all the cholesterol gallstones. Perhaps, Moynihan, widely perceived as the father of modern biliary surgery, was correct when he opined that "each gallstone is a tomb stone erected in memory of a bacterium within". However, studies using more accurate tests on larger patient and control groups are needed to ascertain whether these microorganisms are innocent bystanders or active participants in cholesterol gallstone formation.

## **CORRESPONDENCE TO**

Prof. DHANANJAYA SHARMA MS, PhD (GI Surgery), DSc (GI Surgery) Head, Department of Surgery Government Medical College and Allied Hospitals JABALPUR (MP) INDIA 482 003 +91-761-400 4069 (FAX) +91-94251 56445 (MOBILE) +91-761-2671 522, +91-761-2671 533 (RESIDENCE) e-mail = dhanshar@gmail.com

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**Author Information**

**Nishant Khare, MS**

Department of Surgery, Government Medical College

**Dhananjaya Sharma, MS, PhD, DSc**

Department of Surgery, Government Medical College

**Uday Somashekar, MS, DNB**

Department of Surgery, Government Medical College

**Advait Prakash, MS**

Department of Surgery, Government Medical College

**S. Prakash, PhD**

Entomology Division, Defense Research and Development Establishment

**MJ Mendki, PhD**

Entomology Division, Defense Research and Development Establishment

**Anup Anvikar, MD**

Regional Medical Research Centre for Tribals, Indian Council of Medical Research