

# World Journal of *Gastroenterology*

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**EDITORIAL**

- 1 Estrogen, estrogen receptors, and hepatocellular carcinoma: Are we there yet?  
*Sukocheva OA*

**REVIEW**

- 5 Relationship between intestinal microbiota and ulcerative colitis: Mechanisms and clinical application of probiotics and fecal microbiota transplantation  
*Shen ZH, Zhu CX, Quan YS, Yang ZY, Wu S, Luo WW, Tan B, Wang XY*

**MINIREVIEWS**

- 15 Updated review on immune factors in pathogenesis of Crohn's disease  
*Li N, Shi RH*

**ORIGINAL ARTICLE****Basic Study**

- 23 Construction of an oesophageal cancer-specific ceRNA network based on miRNA, lncRNA, and mRNA expression data  
*Xue WH, Fan ZR, Li LF, Lu JL, Ma BJ, Kan QC, Zhao J*

- 35 Emodin and baicalein inhibit sodium taurocholate-induced vacuole formation in pancreatic acinar cells  
*Li J, Zhou R, Bie BB, Huang N, Guo Y, Chen HY, Shi MJ, Yang J, Zhang J, Li ZF*

**Case Control Study**

- 46 Increased intestinal mucosal leptin levels in patients with diarrhea-predominant irritable bowel syndrome  
*Liu DR, Xu XJ, Yao SK*

**Retrospective Cohort Study**

- 58 Correlation between smoking habit and surgical outcomes on viral-associated hepatocellular carcinomas  
*Kai K, Komukai S, Koga H, Yamaji K, Ide T, Kawaguchi A, Aishima S, Noshiro H*

**Retrospective Study**

- 69 Safety and efficacy of metallic stent for unresectable distal malignant biliary obstruction in elderly patients  
*Sakai Y, Iwai T, Shimura K, Gon K, Koizumi K, Ijima M, Chiba K, Nakatani S, Sugiyama H, Tsuyuguchi T, Kamisawa T, Maetani I, Kida M*
- 76 Short- and long-term outcomes following laparoscopic vs open surgery for pathological T4 colorectal cancer: 10 years of experience in a single center  
*Yang ZF, Wu DQ, Wang JJ, Lv ZJ, Li Y*

- 87 Differential analysis of lymph node metastasis in histological mixed-type early gastric carcinoma in the mucosa and submucosa  
*Zhong Q, Sun Q, Xu GF, Fan XQ, Xu YY, Liu F, Song SY, Peng CY, Wang L*

**Observational Study**

- 96 HLA-DQ: Celiac disease vs inflammatory bowel disease  
*Bosca-Watts MM, Minguez M, Planelles D, Navarro S, Rodriguez A, Santiago J, Tosca J, Mora F*

- 104 Surgical specimen extraction *via* a prophylactic ileostomy procedure: A minimally invasive technique for laparoscopic rectal cancer surgery  
*Wang P, Liang JW, Zhou HT, Wang Z, Zhou ZX*

**Prospective Study**

- 112 Characterization of biofilms in biliary stents and potential factors involved in occlusion  
*Vaishnavi C, Samanta J, Kochhar R*

**SYSTEMATIC REVIEWS**

- 124 Systematic review of colorectal cancer screening guidelines for average-risk adults: Summarizing the current global recommendations  
*Bénard F, Barkun AN, Martel M, von Renteln D*

**META-ANALYSIS**

- 139 Probiotic monotherapy and *Helicobacter pylori* eradication: A systematic review with pooled-data analysis  
*Losurdo G, Cubisino R, Barone M, Principi M, Leandro G, Ierardi E, Di Leo A*

**CASE REPORT**

- 150 Long-term survival after gastrectomy and metastasectomy for gastric cancer with synchronous bone metastasis  
*Choi YJ, Kim DH, Han HS, Han JH, Son SM, Kim DS, Yun HY*
- 157 Emergent single-balloon enteroscopy for overt bleeding of small intestinal vascular malformation  
*Chung CS, Chen KC, Chou YH, Chen KH*

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Editorial board member of *World Journal of Gastroenterology*, Alexander Link, MD, PhD, Academic Research, Associate Professor, Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Hospital Magdeburg, Magdeburg 39120, Germany

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## Prospective Study

# Characterization of biofilms in biliary stents and potential factors involved in occlusion

Chetana Vaishnavi, Jayanta Samanta, Rakesh Kochhar

Chetana Vaishnavi, Jayanta Samanta, Rakesh Kochhar, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

ORCID number: Chetana Vaishnavi (0000-0002-3590-5518); Jayanta Samanta (0000-0002-9277-5086); Rakesh Kochhar (0000-0002-4077-6474).

**Author contributions:** Vaishnavi C conceived the original idea and prepared the study design, provided administrative support, supervised the project and collected the data and finalized the manuscript; Kochhar R provided patients' samples and their clinical details; Samanta J analyzed the data statistically with the help of the department of Biostatistics and wrote the draft manuscript; Kochhar R critically revised the draft manuscript; all authors have directly contributed to the study, reviewed and approved the final manuscript for submission.

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**Clinical trial registration statement:** We have registered our clinical trial with the Indian Council of Medical Research, New Delhi, India, and gave them annual reports of the progress of the project on study of organisms responsible for biofilm formation in biliary stents and their molecular characterization.

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**Correspondence to:** Chetana Vaishnavi, MNAMS, MSc, PhD, Professor, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Sector-12, Chandigarh 160012, India. [vaishnavi.chetana@pgimer.edu.in](mailto:vaishnavi.chetana@pgimer.edu.in)  
Telephone: +91-172-2756609  
Fax: +91-172-2744401

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## Abstract

### AIM

To quantify the components in biofilms and analyze the predisposing factors involved in occlusion of biliary stents.

### METHODS

In a prospective study conducted from April 2011 to March 2014 at a tertiary care hospital, all consecutive patients who required endoscopic biliary stent exchange/removal were included. Etiology of the biliary disease was diagnosed by imaging, cytology and on follow-up. Clinical details of patients with biliary stent retrieval were noted. All extracted stents were collected in sterile containers and immediately



processed for quantification of biofilm proteins and polysaccharides. Molecular identification of commonly known and unknown bacteria was performed by polymerase chain reaction and density gradient gel electrophoresis methods.

## RESULTS

Eighty one patients (41 males) with age range of 20-86 years were studied. The underlying causes for stent insertion were bile duct stones ( $n = 46$ ; 56.8%) benign stricture ( $n = 29$ ; 35.8%) and malignancy ( $n = 6$ ; 7.4%) with cholangitis in 50 (61.7%) patients. The retrieved stent sizes were 7 Fr ( $n = 62$ ; 76.5%) and 10 Fr ( $n = 19$ ; 23.5%) with 65 days median insertion duration. Polybacterial consortia were detected in 90.1% of the stents. The most common bacteria identified by polymerase chain reaction alone and/or sequencing were *Pseudomonas* ( $n = 38$ ), *Citrobacter* ( $n = 23$ ), *Klebsiella* ( $n = 22$ ), *Staphylococcus* ( $n = 20$ ), *Serratia* ( $n = 16$ ), *Escherichia coli* ( $n = 14$ ), *Streptococcus* ( $n = 13$ ), *Enterococcus* ( $n = 13$ ), *Aeromonas* ( $n = 12$ ), *Proteus* ( $n = 10$ ) and *Enterobacter* ( $n = 9$ ). Protein concentration according to gender ( $0.547 \pm 0.242$  mg/mL *vs*  $0.458 \pm 0.259$  mg/mL;  $P = 0.115$ ) as well as age  $> 60$  years and  $< 60$  years ( $0.468 \pm 0.295$  mg/mL *vs*  $0.386 \pm 0.238$  mg/mL;  $P = 0.205$ ) was non-significant. However, polysaccharide concentration was significant both according to gender ( $0.052 \pm 0.021$  mg/mL *vs*  $0.049 \pm 0.016$  mg/mL;  $P < 0.0001$ ) and age ( $0.051 \pm 0.026$  mg/mL *vs*  $0.038 \pm 0.016$  mg/mL;  $P < 0.011$ ). Protein concentration in the biofilm was significantly higher ( $0.555 \pm 0.225$  mg/mL *vs*  $0.419 \pm 0.276$  mg/mL;  $P = 0.018$ ) in patients with cholangitis, lower ( $0.356 \pm 0.252$  mg/mL *vs*  $0.541 \pm 0.238$  mg/mL;  $P = 0.005$ ) in the 10 Fr group than the 7 Fr group, and significantly higher ( $0.609 \pm 0.240$  mg/mL *vs*  $0.476 \pm 0.251$  mg/mL;  $P = 0.060$ ) in stents of  $\geq 6$  mo of indwelling time. However presence/absence of cholangitis, size of stent, indication of stent insertion and indwelling time did not affect the quantity of polysaccharide concentration.

## CONCLUSION

Plastic stents retrieved from patients with biliary tract disease showed polymicrobial organisms with higher protein content among patients with cholangitis and those with smaller diameter stents. Longer indwelling duration had more biofilm formation.

**Key words:** Biofilm constituents; Polybacterial profile; Predisposing factors; Underlying causes; Biliary stents

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**Core tip:** This prospective study evaluated the components in biofilms of retrieved biliary stents and analyzed predisposing factors involved in the process. A majority of stents showed growth of polymicrobial consortia. Polymerase chain reaction and sequencing helped to detect several microorganisms in most of the stents. Presence of cholangitis, smaller diameter

of stents and longer indwelling time of stents were associated with higher chance of biofilm formation. To prevent stent occlusion, longer diameter stents with an indwelling time of 3 to 6 mo should be used.

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## INTRODUCTION

Biliary strictures are responsible for severe complications which can be serious or life threatening to the patients<sup>[1]</sup>. Transpapillary endoscopic stent placement helps in the relief of obstructed biliary system by a non-surgical approach in patients with benign or malignant biliary disease<sup>[2,3]</sup>. The natural microbial barrier posed by the sphincter of Oddi is breached when a stent is placed across it and creates a low resistance pathway for colonization by the intestinal microbes<sup>[4]</sup>. Plastic biliary stents often get occluded by biofilms formed due to adhering microorganisms embedded in an exopolysaccharide (EPS) matrix<sup>[5]</sup>. A biofilm is defined as a collection of microbial communities enclosed by a matrix of EPS, separated by a network of open water channels and attached to man-made or natural surfaces. Bacterial biofilms are formed when unicellular bacteria come together to form a community that is attached to a solid surface and get encased in an exopolymeric substance largely comprising of proteins and different extracellular polymers<sup>[6]</sup>. The proposed mechanism of biofilm formation initiates with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequently formation of an EPS matrix to embed the microbial colonies and other "foreign bodies" to give rise to the final mature biofilm<sup>[2,3]</sup>. Biofilms formed inside biliary stents consist of a mixed spectrum of bacterial communities<sup>[2]</sup>. Most of these bacteria, generally coming from the enterocolon, are uncultivable by standard culture methods.

Clinical stent occlusion leads to jaundice and bacterial cholangitis with polymicrobial infections in up to 90% of patients<sup>[7,8]</sup>. Improper use of antimicrobial agents against these microbes leads to antimicrobial resistance and consequently to ineffective treatment of stent-associated cholangitis<sup>[9]</sup>. Moreover occluded stents need repeat procedures and subsequently lead to increased medical costs as well as poor quality of life. Microorganisms isolated from blocked biliary stents include both aerobic and anaerobic species apart from fungi<sup>[2]</sup> and reveal their intestinal origin<sup>[10-12]</sup>. The material properties of the biofilm are heavily dependent on the composition of the EPS which consists of

proteins, polysaccharides, nucleic acids and lipids in varying proportions depending on the milieu in which the biofilm grows<sup>[13]</sup>. Materials derived from bacteria and the host form a conditioning film which lays the foundation for the biofilm development and initiates the process of bacteria-driven sludge formation<sup>[3]</sup>.

In spite of multiple studies on isolation of various organisms in the formation of biofilms, factors involved in the formation of these biofilms are not well studied. Proper characterization of biofilm formation in plastic stents is yet to be adequately explained before steps for its prevention can be made successful. In this study we elucidated (1) the various bacteria in biofilm formation in biliary plastic stents by molecular identification inclusive of polymerase chain reaction (PCR) and sequencing; (2) principal constituents of biofilms viz. proteins and polysaccharides; and (3) the possible predisposing factors in relation to biofilm formation in the stents.

## MATERIALS AND METHODS

### Study population

This was a prospective study conducted over a three year period at a tertiary care hospital in Northern India (Postgraduate Institute of Medical Education and Research, Chandigarh, India) from April 2011 to March 2014. During this period, all consecutive patients who required an elective or emergency biliary stent exchange/removal were included in the study. The study was reviewed and approved by the Institutional Ethics Committee which operates according to the Declaration of Helsinki. Written informed consent was taken from all the patients prior to study enrollment. Clinical details of each patient were noted with reference to age, sex, etiology, presence of cholangitis and duration for which the stents had been *in situ*. Cholangitis was diagnosed as per the Tokyo guidelines<sup>[14]</sup>. Etiology of the biliary disease was diagnosed by imaging, cytology and on follow-up. All the stents had been placed endoscopically earlier in our institution.

### Interventional procedure

Stent exchange or removal was carried out by first confirming the position of the stent under fluoroscopy. Thereafter, the stents were retrieved endoscopically after grasping with sterile foreign body forceps or a snare and withdrawal of the instrument wholly. The retrieved stents were immediately transferred to a sterile container and transported to the Microbiology Division of the department for processing in order to provide good pre-analytic conditions.

### Molecular identification of the bacterial species occluding the biliary stents

For molecular identification of bacterial species, the central part of the biliary stents were cut and divided horizontally under sterile conditions. The encrusted

sludge within the stent was then cultured aerobically in Brain Heart Infusion broth. For identification of anaerobic bacteria, the crust from stents were cultured in Brucella broth under anaerobic conditions. The microbial DNA was extracted from the culture growth by phenol-chloroform method. Briefly 1.5 mL media containing the growth was centrifuged at 12000 g for 10 mins. The supernatant was discarded and the pellet obtained was resuspended in Tris-EDTA (TE). Sodium dodecyl sulfate (0.5%) and proteinase K (200 µg/mL) was added and incubated for 30 min. Next 100 µL sodium chloride was added followed by an equal amount of chloroform-iso-amyl alcohol. The solution was mixed thoroughly and centrifuged. The supernatant was transferred to fresh tube and equal amount of phenol: chloroform: iso-amyl alcohol was added. After mixing again the solution was centrifuged and the supernatant obtained was transferred to another tube to which 0.6 volume of iso-propanol was added. Centrifugation was repeated and the pellet was washed with 70% ethanol. The pellet obtained was air dried, dissolved in TE and run in 0.8% agarose gel for checking for DNA.

### Identification of commonly known bacteria involved in biofilm formation:

PCR was standardized using the universal 16S rRNA gene specific primers for determining the DNA sequence for commonly known bacteria such as *Pseudomonas*, *Escherichia coli*, *Citrobacter*, *Streptococcus*, *Aeromonas*, *Enterococcus*, *Staphylococcus*, *Proteus*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Vibrio*, *Yersinia*, *Bacteroides* and *Clostridium* using standard strains obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, as positive controls. Following standardization, PCR was done for identification of the above known bacteria that could be responsible for biofilm formation in the stents. The primers used for determination of bacteria are given in Table 1. The amplicons were visualized on 1.5% agarose gels stained with ethidium bromide and compared to a database of known sequences.

### Identification of unknown bacteria involved in biofilm formation:

Molecular identification of unknown bacteria involved in biofilm formation was done using the Density Gradient Gel Electrophoresis (DGGE). The DNA isolated from the biofilms were used for creating multiple copies of the 16S rRNA genes of similar but not identical bacteria for identifying unknown bacteria. The variable regions V3 to V5 were amplified using the following universal primers: 341-F (5'-CCT ACG GGA GGC AGC AG-3') with a 40 bp GC sequence clamped to its 5' end (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') and 907-R (5'-CCG TCA ATT CMT TTG AGT TT-3'). This set of primers was designed to be specific for most bacteria<sup>[15]</sup>. The reaction mixture (50 µL) contained 50 ng

Table 1 Primer sequences used for polymerase chain reaction

Sr. No	Organism	Primer sequences
1	<i>Pseudomonas</i>	F: 5'-GACGGGTGAGTAATGCCTA-3' R: 5'-CACTGGTGTTCCTTCCTATA-3'
2	<i>Staphylococcus</i>	F: 5'- AAC TCT GTT ATT AGG GAA GAA CA-3' R: 5'- CCA CCT TCC TCC GGT TTG TCA CC-3'
3	<i>E. coli</i>	F: 5'-GAAGCTTGCTTCTTTGCT-3' R: 5'-GAGCCCGGGGATTTACAT-3'
4	<i>Enterococcus</i>	F: 5'- GTTATGCCGCATGGCATAAGAG-3' R: 5'-CCGTCAGGGGACGTTTCAG-3'
5	<i>Citrobacter</i>	F: 5'-TCAGATTGAACGCTGGCGGCA-3' R: 5'-CGTATTACCGCGGCTGCTGCCAC-3'
6	<i>Proteus</i>	F: 5'-AGA GTT TGA TCC TGG CTC AG-3' R: 5'-AAG GAG GTG ATC CAG CC-3'
7	<i>Klebsiella</i>	F: 5'-AGA GTT TGA TCC TGG CTC AG-3' R: 5'-AAG GAG GTG ATC CAG CC-3'
8	<i>Clostridium</i>	F: 5'-TGG CTC AGA TTG AAC GCT GGC GGC-3' R: 5'-TAC CTT GTT ACG ACT TCA CCA CA-3'
9	<i>Bacillus</i>	F: 5'- AGA GTT TGA TCC TGG CTC AG-3' R: 5'- AAG GAG GTG ATC CAG CCG CA-3'
10	<i>Vibrio</i>	F: 5'-AGA GTT TGA TCA TGG CTC AG-3' R: 5'-GAA ATT CTA CCC CCC TCT ACA G-3'
11	<i>Aeromonas</i>	F: 5'-GCT GGT CTG AGA GGA TGA TC-3' R: 5'-CTT TAC GCC CAG TAA TTC CG-3'
12	<i>Bacteroides</i>	F: 5'- ATT CTA GAG TTT GAT CAT GGC TCA-3' R: 5'-ATG GTA CCG TGT GAC GGG CGG TGT GTA-3'
13	<i>Enterobacter</i>	F: 5'-AGTTTGATCTGGCTCAG-3' R: 5'-TAC CTT GTT ACG ACT TCG TCC CA-3'
14	<i>Streptococcus</i>	F: 5'-TAA CCA GAA AGG GAC GGC TA-3' R: 5'-CAC TCT CCG CTT CTG CAC TC-3'
15	<i>Serratia</i>	F: 5'-GCGGTTTGTTAAGTCAGATG-3' R: 5'-CGAATTAAACCACATGCTCC-3'
16	<i>Yersinia</i>	F: 5'-AAT ACC GCA TAA CGT CTT CG-3' R: 5'-CTT CTT CTG CGA GTA ACG TC-3'

microbial DNA, 200 µmol/L of each deoxynucleoside triphosphate, 0.5 pmol/L of each of the primers, 2.5 mmol/L MgCl<sub>2</sub>, 3 mg/mL BSA and 3 U DNA Taq polymerase. The touchdown PCR was performed in eppendorf thermocycler using a program described by Sánchez *et al.*<sup>[16]</sup>. Following an initial denaturation at 95 °C for 3 min, a touchdown program began with 15 cycles consisting of one minute denaturation at 95 °C, one minute annealing beginning at 65 °C and ending at 50 °C (decreasing 1 °C per cycle), and a one minute extension at 72 °C. A final extension of 5 min at 72 °C was done. PCR products were quantified on 1.5% (w/v) agarose gel. The desired PCR product was 594 bp (including the GC clamp).

The sample was loaded in the DGGE gel solution consisting of 6% (w/v) acrylamide/bisacrylamide (37.5:1) in 0.5 × TAE buffer containing 40% to 60% of the denaturant. The gels were prepared, loaded and run according to the instructions of the manufacturers of DGGE system (D-Code, BioRad, United States) for analysis of PCR products.

For sequencing, the selected DGGE bands were excised from the gels using sterile scalpel and placed in a sterile eppendorf containing 20 µL of sterile water. The amplified PCR products were sequenced commercially (Chromous Biotech, Bengaluru, India) using bands

which were different from commonly known bands. Data obtained after sequencing in fasta format were compared with the National Center of Biotechnology Information (NCBI) GenBank data base using standard nucleotide blast search tools (BLAST N 2.2.29+).

#### Quantification of major molecules in the biofilms

The major molecules like proteins and carbohydrates which act as a molecular glue for biofilm formation were measured as follows.

**Protein estimation:** Protein estimation in the biofilm mass was done by modified Lowry's method as described by Raunkjær *et al.*<sup>[17]</sup>. Briefly, one centimeter of the stent was put into a 1.5 mL centrifuge tube and 500 µL sodium hydroxide (0.5 mol/L) was added to it. The tube with the stent was heated at 80 °C for 30 min in a water bath. Centrifugation at 4238 *g* at 4 °C for 15 min was done and supernatant was transferred to another micro-centrifuge tube. 50 µL supernatant was put into a test tube and 1 mL reagent comprising of CuSO<sub>4</sub>·5H<sub>2</sub>O and sodium tartrate was added. It was incubated for 5 min at room temperature and absorbance was read at 620 nm using a colorimeter (Electronics India). Bovine serum albumin served as the standard for the assay.



**Table 2** Organisms identified by polymerase chain reaction and sequencing (accession Nos. KP198519-43; KP205043-80; KP2)

Sr. No.	Organisms identified by touchdown PCR and sequencing	Organisms identified by PCR alone
BF 1	<i>Stenotrophomonas maltophilia</i>	<i>Klebsiella</i>
BF 2	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas</i> , <i>Proteus</i> , <i>Aeromonas</i>
BF 3	<i>Bacillus tequilensis</i>	<i>Staphylococcus</i> , <i>Bacillus</i>
BF 4	Uncultured bacterium clone DoIRC 17069	<i>Streptococcus</i> , <i>Aeromonas</i> , <i>Serratia</i>
BF 5	<i>Bacillus cereus</i>	<i>Staphylococcus</i> , <i>Bacillus</i>
BF 6	<i>Micrococcus yunnanensis</i>	<i>Streptococcus</i> , <i>Proteus</i> , <i>Serratia</i>
BF 7	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus</i> , <i>Bacillus</i>
BF 8	<i>Citrobacter</i> sp.	<i>Citrobacter</i> , <i>Escherichia coli</i>
BF 9	<i>Stenotrophomonas maltophilia</i>	<i>Proteus</i>
BF 10	<i>Anaerobaculum</i> sp.	<i>Pseudomonas</i> , <i>Citrobacter</i>
BF 11	<i>Enterobacteriales bacterium</i>	<i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Klebsiella</i>
BF 12	Uncultured bacterium clone PS B346	<i>Staphylococcus</i> , <i>Aeromonas</i>
BF 13	<i>Stenotrophomonas maltophilia</i>	--
BF 14	<i>Stenotrophomonas maltophilia</i>	<i>Streptococcus</i>
BF 15	<i>Stenotrophomonas maltophilia</i>	<i>Serratia</i>
BF 16	Uncultured organism clone ELU0026	<i>Citrobacter</i>
BF 17	Uncultured organism clone ELU0020	--
BF 18	<i>Stenotrophomonas maltophilia</i>	<i>Citrobacter</i> , <i>Streptococcus</i>
BF 19	Uncultured bacterium clone ELU0020	<i>Klebsiella</i> , <i>Aeromonas</i> , <i>Enterococcus</i>
BF 20	<i>Bacillus mojavensis</i>	<i>Pseudomonas</i> , <i>Bacillus</i> , <i>Enterobacter</i>
BF 21	<i>Paenibacillus</i> sp. A1006	<i>Proteus</i> , <i>Klebsiella</i> , <i>Serratia</i>
BF 22	<i>Bacillus cereus</i>	<i>Streptococcus</i> , <i>Serratia</i>
BF 23	<i>Stenotrophomonas maltophilia</i>	--
BF 24	<i>Bacillus</i> sp.	<i>Proteus</i> , <i>Yersinia</i> , <i>Aeromonas</i>
BF 25	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas</i> , <i>Escherichia coli</i>
BF 26	<i>Enterobacteriales bacterium</i>	<i>Pseudomonas</i> , <i>Proteus</i> , <i>Klebsiella</i>
BF 27	Uncultured bacterium clone DoIRC DL35rect19C08	<i>Proteus</i> , <i>Klebsiella</i>
BF 28	<i>Enterococcus faecalis</i>	<i>Citrobacter</i> , <i>Enterococcus</i>
BF 29	<i>Micrococcus luteus</i>	--
BF 30	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus</i> , <i>Serratia</i>
BF 31	<i>Staphylococcus epidermidis</i>	<i>Citrobacter</i> , <i>Escherichia coli</i> , <i>Staphylococcus</i>
BF 32	<i>Enterococcus durans</i>	<i>Escherichia coli</i> , <i>Klebsiella</i> , <i>Enterococcus</i>
BF 33	<i>Enterococcus durans</i>	<i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Serratia</i>
BF 34	<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas</i>
BF 35	Uncultured bacterium clone B64	<i>Citrobacter</i> , <i>Proteus</i> , <i>Klebsiella</i>
BF 36	<i>Pseudomonas otitidis</i>	<i>Pseudomonas</i> , <i>Aeromonas</i>
BF 37	<i>Enterobacteriales bacterium</i>	<i>Streptococcus</i> , <i>Klebsiella</i> , <i>Enterobacter</i>
BF 38	<i>Pseudomonas alcaligenes</i>	<i>Citrobacter</i> , <i>Escherichia coli</i> , <i>Yersinia</i>
BF 39	<i>Enterococcus faecalis</i>	<i>Citrobacter</i> , <i>Streptococcus</i> , <i>Klebsiella</i>
BF 40	<i>Enterococcus faecalis</i>	<i>Streptococcus</i> , <i>Klebsiella</i> , <i>Aeromonas</i>
BF 41	<i>Enterococcus</i> sp.	<i>Citrobacter</i> , <i>Enterobacter</i>
BF 42	<i>Bacillus subtilis</i>	<i>Pseudomonas</i> , <i>Streptococcus</i> , <i>Aeromonas</i>
BF 43	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> , <i>Escherichia coli</i> , <i>Yersinia</i>
BF 44	Uncultured bacterium	<i>Pseudomonas</i> , <i>Citrobacter</i> , <i>Yersinia</i>
BF 45	Uncultured bacterium	<i>Pseudomonas</i> , <i>Citrobacter</i> , <i>Escherichia coli</i>
BF 46	<i>Pseudomonas</i> sp.	<i>Escherichia coli</i> , <i>Enterobacter</i>
BF 47	<i>Bacillus cereus</i>	<i>Proteus</i> , <i>Klebsiella</i>
BF 48	<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas</i> , <i>Citrobacter</i> , <i>Streptococcus</i>
BF 49	<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Proteus</i>
BF 50	<i>Bacillus cereus</i>	<i>Pseudomonas</i> , <i>Staphylococcus</i>
BF 51	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> , <i>Citrobacter</i> , <i>Escherichia coli</i>
BF 52	<i>Pseudoxanthomonas icgebensis</i>	<i>Citrobacter</i> , <i>Serratia</i>
BF 53	<i>Enterobacteriales bacterium</i>	<i>Klebsiella</i> , <i>Enterobacter</i> , <i>Vibrio</i>
BF 54	<i>Citrobacter freundii</i>	<i>Citrobacter</i> , <i>Staphylococcus</i>
BF 55	Uncultured bacterium clone PS	<i>Staphylococcus</i> , <i>Serratia</i>
BF 56	<i>Enterobacteriales bacterium</i>	<i>Pseudomonas</i> , <i>Citrobacter</i> , <i>Streptococcus</i>
BF 57	<i>Morganella morganii</i>	--
BF 58	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas</i> , <i>Escherichia coli</i> , <i>Staphylococcus</i>
BF 59	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Serratia</i>
BF 60	<i>Pseudomonas putida</i>	<i>Pseudomonas</i> , <i>Klebsiella</i> , <i>Aeromonas</i>
BF 61	<i>Morganella morganii</i>	--
BF 62	<i>Enterobacteriales bacterium</i>	<i>Pseudomonas</i> , <i>Aeromonas</i>
BF 63	<i>Enterococcus faecalis</i>	<i>Enterococcus</i>
BF 64	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Serratia</i>
BF 65	<i>Bacillus</i> sp.	<i>Citrobacter</i> , <i>Enterobacter</i>
BF 66	Uncultured organism clone	--
BF 67	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i>

BF 68	<i>Bacillus licheniformis</i>	<i>Pseudomonas</i> , <i>Enterobacter</i>
BF 69	<i>Enterococcus</i> sp.	<i>Klebsiella</i> , <i>Serratia</i>
BF 70	<i>Enterococcus faecalis</i>	<i>Citrobacter</i> , <i>Enterobacter</i> , <i>Serratia</i>
BF 71	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Aeromonas</i>
BF 72	<i>Escherichia coli</i>	<i>Escherichia coli</i>
BF 73	<i>Klebsiella</i> sp.	<i>Pseudomonas</i> , <i>Klebsiella</i>
BF 74	Uncultured <i>Klebsiella</i> sp.	<i>Escherichia coli</i>
BF 75	<i>Enterobacteriales bacterium</i>	<i>Escherichia coli</i> , <i>Enterobacter</i> , <i>Yersinia</i>
BF 76	<i>Stenotrophomonas maltophilia</i>	— —
BF 77	<i>Enterobacteriales bacterium</i>	<i>Klebsiella</i>
BF 78	<i>Citrobacter</i> sp. enrichment clone	<i>Citrobacter</i>
BF 79	<i>Serratia marcescens</i>	<i>Staphylococcus</i> , <i>Serratia</i>
BF 80	<i>Klebsiella</i> sp. BAB-3527	<i>Klebsiella</i> , <i>Aeromonas</i>
BF 81	<i>Exiguobacterium aurantiacum</i>	<i>Klebsiella</i>

PCR: Polymerase chain reaction.

**Polysaccharide estimation:** Polysaccharide estimation in the biofilm mass was done using the anthrone method as described by Ahimou *et al.*<sup>[13]</sup>. Briefly, one centimeter of biliary stent was put into 1.5 mL centrifuge tube and 500  $\mu$ L sodium hydroxide (1 N) was added to it and heated at 80 °C for 30 min in a water-bath. Centrifugation was done at 4238 *g* and 500  $\mu$ L supernatant was transferred to another micro-centrifuge to which 500  $\mu$ L distilled water and 4 mL of 0.2% anthrone reagent in concentrated sulfuric acid was added and mixed well. It was incubated for 10 min in boiling water-bath and allowed to cool at room temperature. Glucose (1 mg/10 mL) was used as a standard and absorbance was read at 620 nm using a colorimeter (Electronics India).

### Statistical analysis

Statistical analysis for this study was performed using SPSS version 20.0 (IBM Corp., United States). The distribution of quantitative and qualitative data was presented as median (range) or absolute and relative frequencies.  $\chi^2$  test and Fisher's exact test were used to investigate the relationship between each parameter. Significance was defined as a *P* value < 0.05.

## RESULTS

### Patient and stent characteristics

A total of 81 patients (41 males) with age-range of 20-86 years were included in the study. The underlying causes for stent insertion were bile duct stones (*n* = 46, 56.8%) benign stricture (*n* = 29, 35.8%), and malignant stricture (*n* = 6, 7.4%). All the stents were double pig-tailed and made of polyethylene (Wilson-Cook Medical, Ireland) and had been placed endoscopically at our Institute. The diameter of the stents retrieved was 7Fr (*n* = 62, 76.5%) or 10 Fr (*n* = 19, 23.5%). The median duration of stent insertion was 65 days (range 5-1095 d). Cholangitis was present in 50 (61.7%) patients, at the time of stent insertion.

### Constituents of biofilms

**Microbiological analysis:** Of the 81 stents retrieved,

organisms were detected in 73 by PCR alone, whereas all 81 stents had organisms detected by touchdown PCR and sequencing which included uncultured bacteria in 12 stents (Table 2). Polybacterial consortia were detected in majority of the stents (*n* = 73, 90.1%) whereas single species were found in the remaining 8 (9.9%) stents. The most common Gram-negative bacteria detected by both PCR alone and by sequencing were *Pseudomonas* (*n* = 38), *Citrobacter* (*n* = 23), *Klebsiella* (*n* = 22), *Serratia* (*n* = 16), *Escherichia coli* (*n* = 14), *Aeromonas* (*n* = 12), *Proteus* (*n* = 10), *Enterobacter* (*n* = 9). The most prevalent Gram-positive bacteria were *Staphylococcus* sp. (*n* = 20) *Streptococcus* (*n* = 13) and *Enterococcus* (*n* = 13). Figure 1A and B show amplification of 541 bp of *Pseudomonas* sp and 500 bp of *Citrobacter* sp. as representative bacteria detected by PCR. Table 3 depicts the number of biliary stents in which Gram negative and Gram positive bacteria were detected.

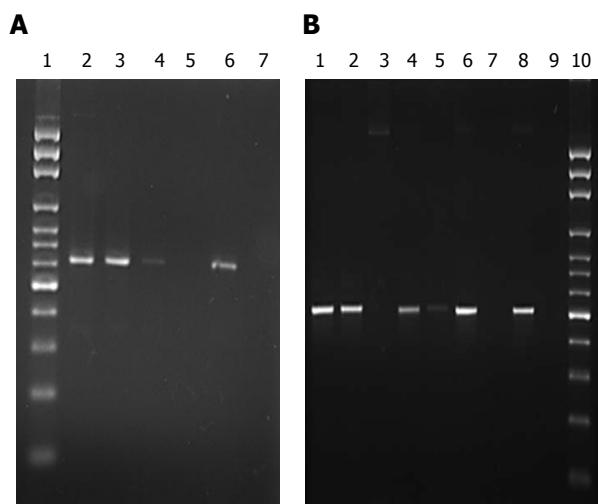
**Quantification of protein and polysaccharide in the biofilms:** Protein content in the biofilm formation ranged from 0 to 0.98 mg/mL with a mean of 0.50  $\pm$  0.25 mg/mL (Figure 2) while the polysaccharide content ranged from 0.014 to 0.107 mg/mL with a mean of 0.051  $\pm$  0.018 mg/mL (Figure 3).

### Relationship of biofilm constituents with predisposing factors

The relation of biofilm constituents with various predisposing factors was analyzed and is summarized in Table 4.

**Gender and age groups:** Male gender had higher protein concentration (*P* = 0.115) and polysaccharide concentration (*P* < 0.0001) than female gender. Patients > 60 years of age had higher protein concentration (*P* = 0.205) and polysaccharide concentration (*P* < 0.011) than those < 60 years of age.

**Cholangitis:** The quantity of biofilm components was compared in the stents retrieved from patients with (*n* = 50) and without cholangitis (*n* = 31). Protein



**Figure 1** 16S rRNA gene (541 bp) of *Pseudomonas* (A) and 16S rRNA gene (500 bp) of *Citrobacter* (B). A: Lane 1: DNA ladder 100 bp; Lanes 2-5: Samples; Lane 6: Positive control; Lane 7: Negative control. B: Lanes 1-7: Samples; Lane 8: Negative control; Lane 9: Positive control; Lane 10: DNA ladder 100 bp.

**Table 3** Microbial species detected from the biliary stents (*n* = 81)

	Number of stents positive for the isolates
Gram-positive microorganism	
<i>Bacillus</i> sp.	11
<i>Enterococcus</i> sp.	9
<i>Micrococcus</i> sp.	2
<i>Streptococcus</i> sp.	11
<i>Staphylococcus</i> sp.	17
Gram-negative microorganism	
<i>Citrobacter</i> sp.	20
<i>Escherichia coli</i>	12
<i>Enterobacter</i> sp.	9
<i>Klebsiella</i> sp.	19
<i>Morganella morganii</i>	2
<i>Proteus</i> sp.	10
<i>Pseudomonas</i> sp.	27
<i>Serratia</i> sp.	15
<i>Stenotrophomonas maltophilia</i>	11
<i>Vibrio</i> sp.	1
<i>Yersinia</i> sp.	5
<i>Aeromonas</i> sp.	12

concentration was found to be significantly higher ( $P = 0.018$ ) in stents with cholangitis (0.555 mg/mL) as compared to those without cholangitis (0.419 mg/mL). Polysaccharide content was however not different in patients with or without cholangitis.

**Indication of stent insertion:** Biofilm constituents were also compared with etiology of biliary disease (benign stricture vs stone). However there was no statistical significance observed between CBD stone or benign stricture as regards to both protein and polysaccharide quantity.

**Size of indwelling stents:** Biofilm constituents were

analyzed between two stent size groups of 7 Fr vs 10 Fr. Protein concentration in the 10 Fr group was significantly lower than in the 7 Fr group ( $0.356 \pm 0.252$  mg/mL vs  $0.541 \pm 0.238$  mg/mL,  $P = 0.005$ ). However there was no significant difference in the quantity of polysaccharide concentration ( $P = 0.674$ ) in the stents of the two different sizes.

**Duration of indwelling stents:** When the stents with an indwelling time of  $\geq 3$  mo were compared with those  $< 3$  mo, it was found that there was no significant difference in the protein concentration ( $P = 0.472$ ) or polysaccharide concentration ( $P = 0.385$ ) between the two groups. When the stents with an indwelling time of  $\geq 6$  mo were compared with those  $< 6$  mo, it was found that protein concentration was significantly higher in the stents of  $\geq 6$  mo of indwelling time ( $0.609 \pm 0.240$  mg/mL vs  $0.476 \pm 0.251$  mg/mL,  $P = 0.060$ ), but there was no difference in polysaccharide concentration ( $P = 0.560$ ).

**Number of microorganisms detected:** When the number of microorganisms isolated *i.e.* single vs multiple by PCR alone was analyzed no significant difference was seen with respect to the protein ( $P = 0.996$ ) and the polysaccharide parameters ( $P = 0.968$ ).

#### GenBank submission

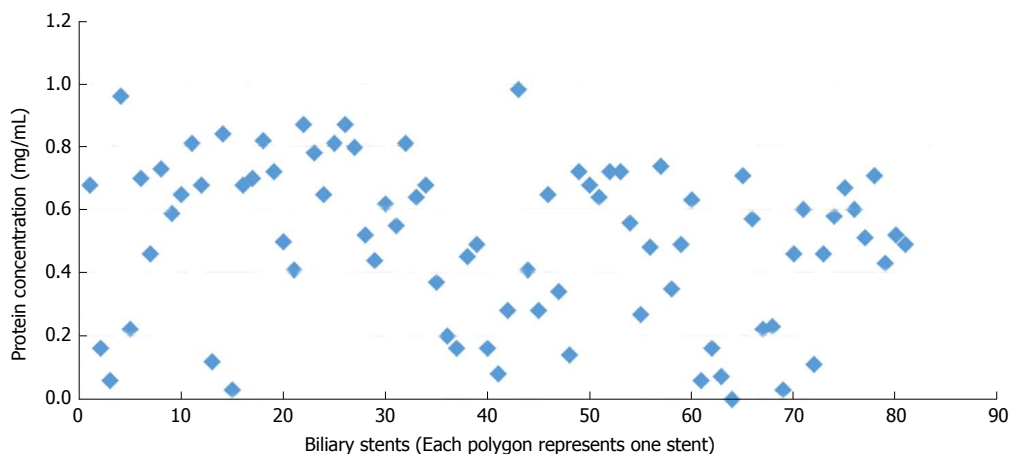
Most of the annotated DNA sequences obtained by sequencing from biofilms of each biliary stent have been deposited with the GenBank at NCBI, United States (Accession Nos. KP198519-43; KP205043-80; KP212173-77).

## DISCUSSION

Biofilm formation is an important step in the occlusion of biliary stents and depends on a number of factors, inclusive of bacterial colonization<sup>[2,3]</sup>. Swidsinski *et al.*<sup>[18]</sup> had demonstrated that neither the gall bladder wall nor the bile duct wall had any biofilm, denoting that opportunistic attachment of the microbes occurs later with subsequent biofilm formation on the biliary stents. In the natural setting, bacteria composed of a single species are seldom found in biofilms and most of the biofilms are multispecies consortia with a synergistic effect on the biofilm formation<sup>[19]</sup>. Aerobic *Enterococcus*, *E. coli* and *Klebsiella* as also anaerobic *Clostridia* are the most common microorganisms isolated from biliary sludge<sup>[2,20]</sup>. In our study, polybacterial consortia were seen in 90.1% of the biliary stents with most common microorganisms being *Pseudomonas*, *Citrobacter*, *Klebsiella*, and *Staphylococcus*. Similar frequency of polymicrobial consortia was found in patients with or without cholangitis. Schneider *et al.*<sup>[20]</sup> also reported that occluded stents have higher proportion of *Staphylococcus* sp. as compared to the non-occluded ones. Lübbert *et al.*<sup>[9]</sup> reported that enterococci plays a

**Table 4** Various factors in relation to protein and polysaccharide concentrations

Parameters	Protein (mg/ml)	P value	Polysaccharide concentration (mg/mL)	P value
Gender				
Male (n = 41)	0.547 ± 0.242	0.115	0.052 ± 0.021	< 0.0001
Female (n = 40)	0.458 ± 0.259		0.049 ± 0.016	
Age				
Below 60 (n = 60)	0.386 ± 0.238	0.205	0.038 ± 0.016	0.011
Above 60 (n = 21)	0.468 ± 0.295		0.051 ± 0.026	
Etiology of stenting				
Cholangitis (n = 50)	0.555 ± 0.225	0.018	0.0512 ± 0.021	0.790
No cholangitis (n = 31)	0.419 ± 0.276		0.050 ± 0.014	
Indication of stent insertion				
CBD stone (n = 46)	0.518 ± 0.256	0.530	0.051 ± 0.022	0.785
Benign stricture (n = 29)	0.453 ± 0.256		0.050 ± 0.012	
Indwelling stent size				
7 Fr (n = 62)	0.541 ± 0.238	0.005	0.049 ± 0.015	0.674
10 Fr (n = 19)	0.356 ± 0.252		0.052 ± 0.020	
Duration of indwelling stents				
< 3 mo (n = 39)	0.481 ± 0.242	0.472	0.0489 ± 0.015	0.385
≥ 3 mo (n = 42)	0.523 ± 0.264		0.0525 ± 0.022	
< 6 mo (n = 65)	0.476 ± 0.251	0.060	0.0501 ± 0.017	0.560
≥ 6 mo (n = 16)	0.609 ± 0.240		0.0533 ± 0.026	
No of microorganisms detected monomicrobial (n = 13)	0.502 ± 0.263	0.996	0.051 ± 0.018	0.968
Polymicrobial (n = 68)	0.501 ± 0.050		0.049 ± 0.015	

**Figure 2** Protein concentration in biofilms of the biliary stents (n = 81).

significant part in the microbial colonization of biliary stents. In our study enterococci were found in 16% of the biliary stents. Though anaerobes are reported by some authors to have important role in the formation of biofilms<sup>[20]</sup>, we found no anaerobe in the occluded biliary stents in the present study.

The proposed mechanism of biofilm formation is initiated with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequent formation of an EPS matrix to embed the microbial colonies and other particles to give rise to the final mature biofilm. Yu *et al.*<sup>[21]</sup> reported attachment of fibronectin to the inner surface of the stents within 24 h of exposure to bile. Another contributing factor is the bile immunoglobulin-bacteria complex which further

promotes the binding of the bacteria to the inner surface of the stents<sup>[22]</sup>. Thus, the basic ingredients of a biofilm include the adherence proteins, the bacteria and the EPS. In patients with cholangitis, wherein these factors are expected to be high, there are higher chances of biofilm formation and stent occlusion. In the current study, the protein concentration of the biofilms was found to be significantly higher in stents placed in patients with cholangitis than those without cholangitis. Polysaccharide concentration was also higher among the cholangitis group, although it was not statistically significant. This highlights the phenomenon of higher propensity of stent occlusion due to biofilm formation in an infected biliary system as compared to the non-infected ones. The higher risk of stent occlusion in



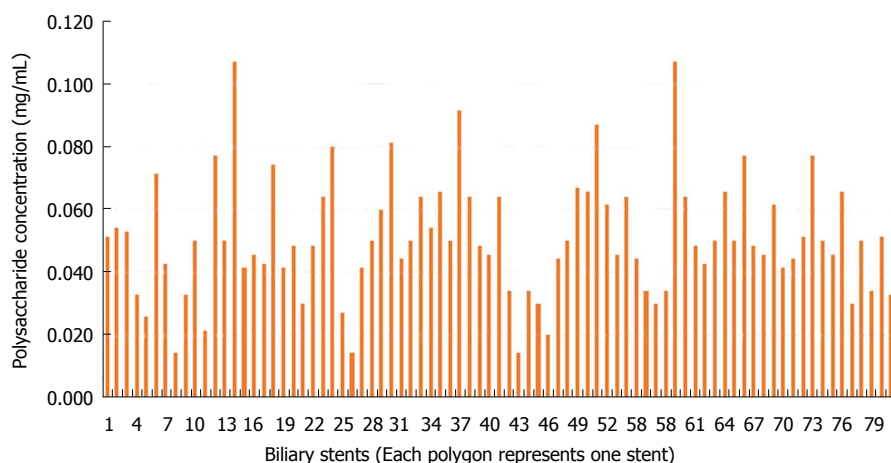


Figure 3 Polysaccharide concentration in biofilms of the biliary stents ( $n = 81$ ).

cholangitis can also be explained by the increased bile viscosity of the infected bile, causing decrease in the bile flow velocity leading to bile stasis and increased spontaneous and bacteria-driven bile salt precipitation<sup>[2]</sup>.

The diameter of the biliary stents has always been a key issue governing the dynamics of bile flow and stent occlusion. An increase in the inner stent diameter of 0.2 mm leads to a 300% increase in the bile flow<sup>[23]</sup>. The maximum diameter of plastic stent that can be placed endoscopically is 11.5 Fr<sup>[24]</sup>. This limitation of the maximum diameter of stents placed endoscopically is the reason why stents up to 10 Fr size are used. Smaller diameter stents have a higher tendency to get occluded due to biofilm formation. Larger diameter entails greater bile flow velocity and subsequently less predisposition to bile salt precipitation, protein accumulation and biofilm formation. Thus, large diameter stents have always fared better than smaller diameter ones in terms of durability<sup>[25]</sup> and one of the major advantages of metallic stents is in fact its large diameter<sup>[26]</sup>. In the current study, 10 Fr stents were found to have significantly lower protein concentration in their biofilm formation as compared to the 7 Fr ones. This highlights a probable lower propensity for protein deposition - one of the key events for initiation of biofilm formation due to high bile flow velocity in the 10 Fr groups.

The process of biofilm formation is a time-dependent one and risk of standard polyethylene stent occlusion increases progressively after 3 mo<sup>[2]</sup>. We did not find any difference in the protein and polysaccharide concentration in relation to stents removed < 3 mo and  $\geq$  3 mo. However when the stents placed for < 6 mo were compared with those of  $\geq$  6 mo, the protein concentration was found to be higher in stents kept for  $\geq$  6 mo. The nature of protein (human/bacterial origin, immunoglobulins, fibrinogen *etc.*) could also not be further analyzed as this was not the aim of the study. Quantitative assessment of the number of

bacteria present in the stents could not be made as molecular identification was carried out after culture of bacteria from the stent segments in fluid culture media. The process of biofilm formation in general is very complex (obstruction of biliary stents is more complex and involves not only bacteria and their products but bilirubin complexes, cholesterol complexes and ingrowth of tissue). Schneider *et al.*<sup>[20]</sup> in a multivariate analysis have shown that sludge formation had significant relationship with stent indwelling time.

Understanding pathophysiology of biofilm formation in plastic biliary stents is important in preventing their occlusion and complications thereof. From our data stents indwelling time of  $\leq$  3 mo or > 3 mo did not correlate with biofilm formation. However stents placed for > 6 mo had higher biofilm formation. Hence stents should not be left indwelling beyond 6 mo. Also larger diameter stents (10 Fr) should be preferred. A number of other options have been studied to prevent biofilm formation. Several studies have shown the effects of antibiotic coatings on medical devices effective against biofilm formation; however, such data has not been as successfully replicated for biliary stents<sup>[27]</sup>. Recently some workers have found that biliary plastic stents coated with silver nano particles or ions have antibacterial activity against several organisms and extends the period of use of biliary stents<sup>[28,29]</sup>.

Our study had a few limitations. Data on comorbidities were not available, so we could not study the predisposition, if any, of biofilm formation in patients with diabetes. We had only a few patients with malignancy who were excluded from analysis. A larger number of patients with malignant obstruction could have given us a comparison between benign and malignant etiology. Culture results of patients with cholangitis at the time of stent insertion were not available to correlate with organisms grown in the biofilms.

Ours is one of the first studies of its kind to measure the biofilm components, namely protein and

polysaccharide in biliary stents. Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer ( $\geq 6$  mo) indwelling time of stents was associated with higher biofilm formation and protein concentration elucidating the time-dependent process of biofilm formation. Our data suggest that plastic stents should be replaced between 3-6 mo.

## ARTICLE HIGHLIGHTS

### Research background

Since its introduction in 1979, biliary plastic stents have been a landmark achievement in the field of endoscopic retrograde cholangiopancreatography for the relief of obstructed biliary system by a non-surgical approach. The limiting factors for these plastic stents are their diameter and the tendency to get occluded. The maximum diameter of plastic stent that can be placed is 11.5 Fr requiring a duodenoscope accessory channel diameter of 4.2 mm. This limitation of the maximum diameter leads to the tendency for them to get occluded due to the formation of biofilm causing recurrent obstruction and need for repeat procedures subsequently leading to increased medical costs and poor quality of life. The cardinal step in the process of stent occlusion is bacterial colonization. Various studies including scanning electron microscopic observations have shown that the clogging material found in biliary stents consists of bacterial biofilm, biliary sludge and duodenal refluxate of dietary fibers. Biofilm is formed by microbes embedded in an exopolysaccharide matrix which also engulfs "foreign bodies" of various sizes. Its ultrastructure reveals voids and channels required for nutrient diffusion and molecular signaling.

### Research motivation

Despite multiple studies elucidating the various organisms and the formation of biofilms, various factors involved in the formation of these biofilms are not well studied. The proposed mechanism of biofilm formation initiates with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequently formation of an exopolysaccharide matrix to embed the microbial colonies and other "foreign bodies" to give rise to the final mature biofilm. However, proper characterization of biofilm formation in plastic stents has to be adequately elucidated before steps for its prevention can be made successful. Components of the biofilm such as protein and polysaccharides developing in biliary stents have never been quantified in previous studies.

### Research objectives

The main objectives of this study were to elucidate the various bacteria implicated in biofilm formation in biliary plastic stents, to quantify the principal constituents (namely proteins and polysaccharide) of biofilm mass and the possible predisposing factors in relation to biofilm formation in the stents. This prospective study evaluated the extracellular polymeric substance such as protein and polysaccharide in the biofilms as well as microbes occluding the biliary stents in patients who had retrieval of biliary stents (7 Fr and 10 Fr) and analyzed predisposing factors involved in the process of occlusion of the stents. Our results showed that the presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer ( $\geq 6$  mo) indwelling time of stents was associated with higher biofilm formation and protein concentration, elucidating the time-dependent process of biofilm formation. Our data suggest that plastic stents should be replaced between 3-6 mo. Further studies can be done to explore the origin of the bacteria grown in biofilms. Strategies to prevent biofilm formation can also be planned and investigated.

### Research methods

This was a prospective study conducted at a tertiary care hospital in Northern India (Postgraduate Institute of Medical Education and Research, Chandigarh, India) from April 2011 to March 2014. All consecutive patients who required an elective or emergency biliary stent exchange/removal were enrolled and clinical details of each patient were noted. The stents were retrieved through video duodenoscope and transferred into sterile containers for processing. For molecular identification of bacterial species, the encrusted material enclosed within the stent was cultured aerobically and anaerobically and the microbial DNA was extracted. PCR was standardized using the universal 16S rRNA gene-specific primers for determining the DNA sequence for commonly known bacteria. Molecular identification of unknown bacteria involved in biofilm formation was done using the Density Gradient Gel Electrophoresis. The amplified PCR products were sequenced commercially using bands which were different from commonly known bands. Data obtained after sequencing were compared with the National Center of Biotechnology Information GenBank data base, using standard nucleotide blast search tools. The major molecules in the biofilms such as protein and polysaccharide were estimated in the biofilm mass by modified Lowry's method and anthrone method respectively. The outcome measures were quantification of biofilm protein, polysaccharides and the organisms and their relation with gender, age, etiology of biliary diseases, stent indwelling time, stent size and the presence of cholangitis. Statistical analysis for this study was performed using SPSS version 20.0 using  $\chi^2$  test and Fisher's exact test to investigate the relationship between various parameters.

### Research results

Higher protein concentration in the biofilm was noted in patients with cholangitis as compared to those without cholangitis. Cholangitis and protein concentration increased the likelihood of biofilm formation in these patients explaining higher stent occlusion rates in infected bile. Male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Smaller diameter stents depicted higher protein concentration predisposing to early biofilm formation thereby indicating the use of larger diameter stents. 10 Fr stents had lower concentration of protein deposition in the biofilm compared to 7 Fr stents and hence explains the longer patency rates. PCR and sequencing helped to detect several commonly known and unknown microorganisms in most of the stents. Time dependent process of biofilm formation was demonstrated by greater quantity of biofilm mass deposition on increasing length of stent indwelling time. Longer indwelling time of stents has a greater likelihood of accumulating higher biofilm formation and patients should be followed-up between 3-6 mo to avoid complications.

### Research conclusions

Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer indwelling time of stents was associated with higher biofilm formation and protein concentration elucidating the time-dependent process of biofilm formation. Our study suggests that plastic stents should be replaced between 3-6 mo. Plastic stents retrieved from patients with biliary tract disease showed polymicrobial organisms with higher protein content among patients with cholangitis and those with smaller diameter stents. Longer indwelling duration had more biofilm formation. Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher tendency of biofilm formation. Longer indwelling stent duration was associated with higher biofilm formation and protein concentration, revealing the time-dependent progression of biofilm formation. Longer indwelling time of stents, smaller diameter stents, male gender and age above 60 years are associated with more biofilm formation. Data on comorbidities such as diabetes in patients should be checked for predisposition, if any, of biofilm formation. Culture results of patients with cholangitis at the time of stent insertion will help to correlate with organisms grown in the biofilms. Protein concentration in the biofilm was significantly higher in patients with cholangitis, lower in the 10 Fr group than the 7 Fr group, and significantly higher in stents of  $\geq 6$  mo of indwelling time. Polysaccharide concentration in biofilms of stents of male gender as well as

in patients with age > 60 years was significant. The most common bacteria identified by PCR alone and/or sequencing were *Pseudomonas* ( $n = 38$ ), *Citrobacter* ( $n = 23$ ), *Klebsiella* ( $n = 22$ ), *Staphylococcus* ( $n = 20$ ), *Serratia* ( $n = 16$ ), *Escherichia coli* ( $n = 14$ ), *Streptococcus* ( $n = 13$ ), *Enterococcus* ( $n = 13$ ), *Aeromonas* ( $n = 12$ ), *Proteus* ( $n = 10$ ) and *Enterobacter* ( $n = 9$ ). Longer indwelling time of stents and smaller diameter stents are associated with more biofilm formation. Larger diameter (10 Fr) stents should be preferred for the relief of obstructed biliary system by a non-surgical approach in patients with benign or malignant biliary disease. Stents should not be kept *in-situ* for more than 3-6 mo.

### Research perspectives

Our study suggests that 10 Fr stents should be preferred over the 7 Fr stents and stents should be replaced between 3-6 mo. Attempts to prevent biofilm formation should be investigated. Ultrastructural characterization of biofilms in occluded stents should also be done.

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