RESEARCH ARTICLE

Microbial biofilms associated with biliary stent clogging

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Abstract

Endoscopic stenting is a palliative approach for the treatment of diseases involving biliary obstruction. Its major limitation is represented by stent occlusion, followed by life-threatening cholangitis, often requiring stent removal and replacement. Although it has been suggested that microbial colonization of biliary stents could play a role in the clogging process, the so far available data, particularly on the role of anaerobic bacteria, are not enough for a comprehensive description of this phenomenon. Our study was focused on the analysis of 28 explanted biliary stents by culturing, denaturing gradient gel electrophoresis and scanning electron microscopy to identify all the aerobic/anaerobic bacteria and fungi involved in the colonization of devices and to verify the ability of isolated anaerobic bacterial strains to form a biofilm in order to better understand the mechanisms of stent clogging.

Introduction

The endoscopic insertion of plastic stents represents an effective system of biliary decompression contributing to the regression of symptomatology and determining a significant improvement of quality of life in patients suffering from obstructive jaundice associated with malignant hepatobiliary tumors or benign strictures (Ballinger *et al.*, 1994). However, the major limitations of this palliative approach are mainly represented by stent occlusion, often followed by life-threatening cholangitis necessitating repeated interventions and stent exchange. Stent occlusion is caused by the deposition of biliary sludge, which is composed of cholesterol crystals, calcium bilirubinate and palmitate, amounts of cholesterol as well as bacteria and/or fungi, microbial byproducts, proteins, dietary fibers and glycoproteins (Dowidar *et al.*, 1991; McAllister *et al.*, 1993; Weickert *et al.*, 2001; Moss *et al.*, 2006; Donelli *et al.*, 2007). Deposition of calcium salts due to the biochemical activities of bacterial enzymes in the biofilm growing on the surface of the stents and reflux of intestinal contents into stents have been proposed as the two main mechanisms of stent occlusion (Speer *et al.*, 1988; Moesch *et al.*, 1991; Sung *et al.*, 1993).

However, some authors suggested that microbial adhesion and biofilm formation on the surface of the stent lumen could play an important role in the initiation of the clogging process and in the subsequent stent blockage (Leung *et al.*, 1988, 2000; Basoli *et al.*, 1999; Di Rosa *et al.*, 1999; van Berkel *et al.*, 2000, 2005; Guaglianone *et al.*, 2008).

Microorganisms gain access into the biliary system either by descending via the portal venous circulation or by ascending through the sphincter of Oddi in duodenal–biliary reflux (Sung *et al.*, 1992). Bacteria adhere to the stent surface and their sessile growth and exopolysaccharide production lead to the establishment of a thick biofilm conferring microorganisms with an efficient protection from both antibacterial agents and phagocytic cells. The β-glucuronidase and lecithinase (or phospholipase C) enzymatic activities of colonizing microorganisms lead to the precipitation of calcium bilirubinate and palmitate, thus contributing to the sludge accumulation within the biliary system and then to the stent occlusion (Leung *et al.*, 1988).

The aims of this study were to analyze the biliary sludge of 28 clogged stents to check the presence of *ex vivo* biofilm formation, to identify all the microbial species colonizing the stents' lumen and to verify the *in vitro* ability of isolated anaerobic bacteria to form a biofilm.

Materials and methods

Patients and stents

Twenty-eight clogged biliary stents were removed from patients (mean age = 66 years) who had undergone endoscopic stent insertion for the treatment of a variety of diseases involving biliary obstruction. The implantation time ranged from 20 to 484 days (mean = 164). The antibiotic therapy received by most of the patients (21/28) in the last 2 months before stent removal included amikacin, amoxicillin/clavulanic acid, ampicillin/sulbactam, ceftriaxone, ciprofloxacin, piperacillin/tazobactam and, only in one patient, metronidazole.

Immediately after removal, segments of approximately 3 cm each were cut under sterile conditions from the distal, central and proximal portions of the stents (Fig. 1), placed into sterile tubes and sent to the laboratory for further processing by scanning electron microscopy (SEM) observation, culture and PCR-denaturing gradient gel electrophoresis (DGGE). This last technique was used to identify, in a random selected sample representing the 50% of all explanted stents, species not recovered by culture.

Microbiological analysis

For the isolation and identification of aerobic bacteria and fungi, the segments obtained from the distal end (A) of stents were bisected along their long axis, placed into sterile phosphate-buffered saline (PBS) (pH 7.4) and sonicated in ice for 10 min at 2 μA (Soniprep 150, MSE). Then 0.1 and 0.01 mL of the suspension were plated on nonselective media and incubated at 37 °C for 24–48 h under aerobic conditions. Isolated microorganisms were counted and identified at the species level using standard biochemical tests.

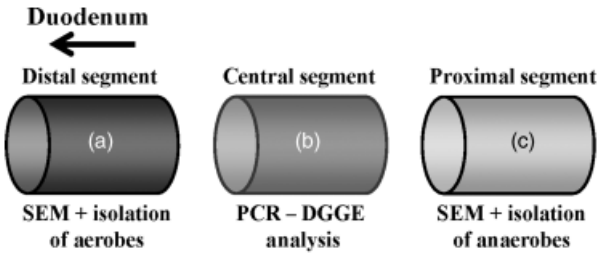

Fig. 1. Scheme representing the stent sectioning according to the analytical approaches performed.

For the isolation and identification of anaerobic bacteria, all procedures were performed in an anaerobic cabinet. Each segment of the proximal portion of the stents was bisected along its major axis and the inner luminal surface of one section of the stent was scraped with a sterile wire loop to remove the sludge and adherent bacteria. Then, the suspension was serially diluted (1 : 10) in sterile PBS and 100 mL of each dilution was spread on prereduced Columbia agar plates supplemented with 5% sheep blood, 0.1% vitamin K1 and hemin and incubated anaerobically at 37 °C for 72 h. The other half of the stent was transferred into prereduced brain–heart infusion broth, vortex mixed and incubated anaerobically for 7 days. After appropriate dilutions, samples were streaked onto Columbia blood agar plates to determine the bacterial density (CFU) and to recover fastidious anaerobes not grown directly on plates. Individual colonies were selected on the basis of their morphology and plates were both aerobically and anaerobically incubated to exclude the aerobic growths. All anaerobes were identified using the RAPID ID 32A kit (BioMérieux).

PCR-DGGE

Each central portion (B) of the biliary stents to be analyzed was bisected along its major axis and the sludge contained in the stent lumen was resuspended in 1 mL of TE buffer (10 mM Tris-HCl, pH 7.2; 1 mM EDTA). The total microbial DNA was directly extracted from the samples according to the method described by Bollet *et al.* (1991). The universal PCR primers U968-f (5′-AAC GCG AAG AAC CTT AC-3′) and L1401-r (5′-GCG TGT GTA CAA GAC CC-3′) were used to amplify the V6–V8 regions of eubacterial 16S rRNA gene (Randazzo *et al.*, 2002), while the universal fungal primers U1 (5′-GTG AAA TTG TTG AAA GGG AA-3′) and U2 (5′-GAC TCC TTG GTC CGT GTT-3′) were used to amplify a portion of 28S rRNA gene (Sandhu *et al.*, 1995). A GC clamp was attached to the 5′-end of the forward primers (Muyzer & Smalla, 1998; Walter *et al.*, 2001). For the 16S rRNA and the 28S rRNA genes, the PCR amplification conditions described by Randazzo *et al.* (2006) and Meroth *et al.* (2003), respectively, were utilized. All the amplifications were performed in a 9700 Gene Amp PCR System (Applied Biosystem). The presence of amplicons was initially assessed by 1.5% w/v agarose gel (Euroclone) electrophoresis in 0.5 × TBE. The PCR products were analyzed by DGGE using the Dcode apparatus (Bio-Rad Laboratories Inc.), according to the procedure described by Cocolin *et al.* (2001). The amplicons obtained with the U968-f-L1401-r primers were electrophoresed for 8 h using a gel containing a 50–70.6% urea-formamide denaturing gradient (100% denaturing solution corresponded to 40% v/v formamide and 7 M urea), while the amplicons obtained with U1–U2 primers were electrophoresed for 4.5 h using gels containing

a 40–60% urea-formamide denaturing gradient. The gels were subjected to a constant voltage of 130 V at 60 °C. After electrophoresis, the gels were stained for 20 min in 1.25 × TAE buffer (50 mM Tris-HCl, 25 mM acetic acid, 1.25 mM EDTA, pH 8.0) containing ethidium bromide solution (10 mg mL^{-1}), rinsed in distillate water and photographed under UV illumination.

The DGGE bands to be sequenced were excised from the gels with sterile scalpels. The DNA was eluted with 50 μL TE buffer and incubated overnight at 4 °C. DNA (6 μL) eluted from each DGGE band was used for amplification using the forward primer without the CG clamp, further purified using the GFX-PCR-DNA and Gel Band purification kit (GE Healthcare, Buckinghamshire, UK) and sent to M-Medical/MWG Biotech (Milan, Italy) for sequencing. The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschul *et al.*, 1997). The lowest percentage of similarity accepted for identification was fixed at 96%.

In vitro biofilm growth of anaerobic isolated strains

The ability of all the anaerobic strains isolated from biliary stents to form biofilm *in vitro* was preliminarily tested by the slime-production assay as described previously (Donelli *et al.*, 2004). Briefly, bacteria were grown anaerobically in prereduced triptic soy broth (TSB) supplemented with 1% glucose overnight at 37 °C. Polystyrene 96-well tissue-culture plates (Corning Costar) were filled with 180 μL of fresh TSB, and 20 μL of the overnight culture was added to each well. The plates were incubated anaerobically for either 8 or 18 h at 37 °C. After incubation, the culture medium was discarded and wells were washed carefully three times with 200 μL of PBS without disturbing the biofilm on the bottom of the wells. The plates were dried for 1 h at 60 °C and stained with 2% Hucker's crystal violet for 2 min. Excess stain was removed by rinsing the plates under tap water, and plates were dried for 10 min at 60 °C. The $OD_{595\,nm}$ was determined in an ELISA reader. Each assay was performed at least in triplicate and repeated at least twice. The $OD_{570\,nm}$ of the biofilm was measured in a spectrophotometer (Novapath Microplate Reader; Bio-Rad Laboratories Inc.). The slime index was defined as an estimate of the density of the biofilm generated by a culture with an $OD_{600\,nm}$ of 0.5 [slime index = mean OD of the biofilm × (0.5/mean OD growth)].

Bacterial isolates resulted to be slime producers, were grown anaerobically on glass coverslips placed on the bottom of 24-well plates containing prereduced TSB supplemented with 1% glucose and incubated for 24 h at 37 °C.

SEM

Segments cut from the distal and proximal parts (A+C) of stents and bisected as described above were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% ruthenium red (Sigma) at room temperature for 30 min. Following postfixation in 1% OsO_4 for 20 min, samples were dehydrated through graded ethanols, critical point dried in hexamethyldisilazane (Polysciences Inc., Warrington, PA), gold coated by sputtering and examined using a Cambridge 360 SEM.

For SEM observation, biofilms grown on coverslips were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 30 min, then postfixed in 1% OsO_4 for 20 min and dehydrated through graded ethanols. After critical point drying in hexamethyldisilazane and gold coating by sputtering, biofilm samples were observed by SEM.

Results

Microorganisms grew from all the 28 examined stents. In particular, on a total of 106 microbial strains, aerobes were isolated from 93%, anaerobes from 57% and fungi from 25% of the samples. The overall results are summarized in Table 1, in which the number of isolated strains belonging to the different species is reported. As better evidenced in Fig. 2, the enterococci were the most frequently occurring species, followed by the Gram-negative bacteria *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp.

Fungi were only represented by *Candida* spp. and were isolated in 25% of the analyzed stents.

Bacteroides spp. and *Clostridium* spp. were the most represented anaerobic species, followed, in order of incidence, by *Prevotella* spp., *Veillonella* spp., *Fusobacterium* spp. and *Peptostreptococcus* spp.

Most of the stents were found to be colonized by more than one microorganism. In fact, 1/28 stents was colonized by only one strain (*Bacteroides capillosus*), while the others were colonized by microbial strains belonging up to six different species, both aerobic and anaerobic.

PCR-DGGE analysis, performed on 13 stent segments belonging to the central portion (B), allowed the identification of a number of bacterial and fungal species (Table 2) in addition to those isolated using cultivation procedures. Among the anaerobes, *Atopobium rimae*, *Bifidobacterium breve*, *Bilophila wadsworthia*, *Mogibacterium diversum* and *Peptostreptococcus stomatis* were additionally identified, while, among aerobes, *Enterobacter aerogenes*, *Enterobacter cancerogenus*, *Enterobacter hormaechei*, *Escherichia albertii*, *E. coli* O157:H7, *Gemella sanguinis*, *Granulicatella* spp., *Morganella morganii* ssp. *morganii*, *Pantoea ananatis*, *Pantoea eucalypti*, *Raoultella terrigena*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei* were also identified.

Table 1. Number of aerobic and anaerobic bacterial and fungal strains, belonging to different species, isolated from the 28 analyzed biliary stents

Gram-positive bacteria	*n*	Gram-negative bacteria	*n*
Aerobes			
Enterococcus faecalis	12	*Escherichia coli*	15
Enterococcus spp.	6	*Pseudomonas aeruginosa*	8
Enterococcus faecium	4	*Pseudomonas* spp.	3
Enterococcus gallinarum	1	*Klebsiella pneumoniae*	5
Coagulase-negative staphylococci	1	*Klebsiella oxytoca*	3
Streptococcus anginosus	1	*Klebsiella ozaenae*	3
		Klebsiella spp.	2
		Enterobacter cloacae	4
		Enterobacter aerogenes	1
		Aeromonas hydrophila	2
		Proteus spp.	2
		Providencia stuartii	2
		Citrobacter amalonaticus	1
		Citrobacter freundii	1
		Hafnia alvei	1
		Serratia liquefaciens	1
Anaerobes			
Clostridium fallax	2	*Bacteroides fragilis*	2
Clostridium bifermentans	1	*Bacteroides capillosus*	1
Clostridium baratii	1	*Bacteroides distasonis*	1
Clostridium difficile	1	*Bacteroides oralis*	1
Clostridium perfringens	1	*Prevotella bivia*	2
Peptostreptococcus magnus	1	*Prevotella intermedia*	2
		Veillonella spp.	2
		Fusobacterium necrophorum	1
Fungi			
Candida albicans	1		
Candida parapsilosis	1		
Candida spp.	6		

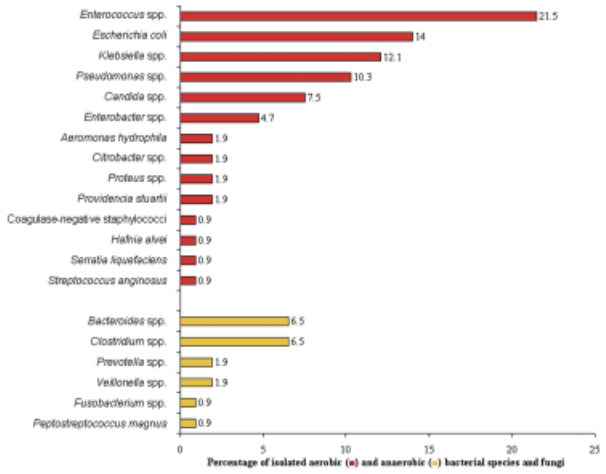

Fig. 2. Frequency of aerobic/anaerobic bacterial and fungal species isolated from explanted biliary stents. Data are based on a total of 106 isolated strains, 79 of which belonged to aerobic bacteria, 19 to anaerobic bacteria and eight to fungi.

Among fungi, *Candida carpophila*, *Candida humilis*, *Candida milleri*, *Kazachstania barnettii* and *Pichia guilliermondii* were additionally identified.

At macroscopical observation (Fig. 3), both the outer (Fig. 3a) and the inner surfaces, obtained by bisecting stents' segments along their longitudinal axis (Fig. 3b), were found to be more or less covered or filled by a yellow brownish, soft and heterogeneous material, respectively.

In Fig. 4, common nonmicrobial sludge components have been observed by SEM including dietary fibers (Fig. 4a), as a result of duodenal reflux, and crystals that were tentatively identified as calcium bilirubinate and calcium palmitate, respectively (Fig. 4b and c).

SEM observation of longitudinal sections of partially occluded stents (Fig. 5) revealed the early phase of sludge formation (Fig. 5a). At a higher magnification, it was possible to recognize coccoid bacterial cells (Fig. 5b), rod-shaped bacteria (Fig. 5c) and fungal cells (Fig. 5d).

Fig. 5c clearly shows the typical appearance of sludge in direct contact with the bile flow as indicated by the mucous material in which bacteria are immersed and grow as a biofilm.

As observed by SEM (Fig. 6), in the cross-section of a stent segment, the dehydration procedures for sample observation frequently caused a cleavage (Fig. 6a) at the interface between the biliary sludge content and the stent lumen. In Fig. 6b, the 'sludge side' of this cleavage is shown in which both coccoid cells and their imprints are observed, while in Fig. 6c, a portion of sludge matrix, devoid of bacteria, but still attached to the lumen surface, can be observed. The sludge detachment from the inner stents' lumen caused by the dehydration procedure evidenced, in almost all samples, clusters of microbial cells closely bound to the polymeric stent surface (Fig. 6d, e and f).

All the 19 isolated anaerobic strains were investigated for their ability to produce slime *in vitro*. Among the 12 Gram-negative anaerobic isolated strains tested for slime production, those belonging to the species *Bacteroides fragilis*, *Fusobacterium necrophorum*, *Prevotella intermedia* and *Veillonella* spp. were strong slime producers, while the strain of *Prevotella bivia* was a weak producer and the three *Bacteroides* strains of *B. capillosus*, *Bacteroides distasonis* and *Bacteroides oralis* were nonproducers (Table 3).

With respect to the six Gram-positive anaerobic strains isolated, five were strong producers (*Clostridium baratii*, *Clostridium perfringens*, *Peptostreptococcus magnus*, *Veillonella* spp. and *F. necrophorum*), two were weak producers (*Clostridium difficile* and *Clostridium fallax*), while only the *Clostridium bifermentans* strain was a nonproducer.

The biofilms formed by four out of seven strong slime-producer strains, after a 24-h incubation, are reported in Fig. 7, in which the typical tridimensional shape of a mature biofilm is clearly evident in all the observed samples.

Table 2. Sequencing results from the bands cut from the DGGE gels

Target region	Sample code	Band(s)*	Closest relative and GeneBank accession no.†		% Identity‡	*E* value
V6–V8 16S region	B 17	1	***Granulicatella adiacens***	AY879305	98	6e − 61
			Granulicatella elegans	EU670066	98	6e − 61
			Granulicatella paraadiacens	AY879301	98	6e − 61
			Streptococcus sobrinus	DQ677791	98	6e − 61
		2	***Mogibacterium diversum***	AB037874	96	2e − 50
	B 19	3	*Escherichia coli*	GQ273519	99	0.0
			***Escherichia coli* O157:H7**	CP001368	99	0.0
			Shigella dysenteriae	FJ937911	99	0.0
			Shigella flexneri	FM207091	99	0.0
			Escherichia albertii	NR_025569	99	0.0
		4	***Morganella morganii* ssp. *morganii***	AF500485	99	0.0
		5	***Pantoea eucalypti***	EF688009	100	3e − 76
			Pantoea ananatis	EU849111	100	3e − 76
			Enterobacteriaceae bacterium	EU040283	100	3e − 76
			Proteus vulgaris	EU373433	100	3e − 76
			Morganella morganii* ssp. *morganii	AF500485	100	3e − 76
	B 20	6	*Escherichia coli*	AM946981	99	0.0
			Shigella sonnei	X80726	99	0.0
		7	***Morganella morganii* ssp. *morganii***	AF500485	99	0.0
		8	*Klebsiella* spp.	FJ984439	99	0.0
			Klebsiella pneumoniae	AP006725	99	0.0
			***Bacillus* spp.**	FJ190415	99	0.0
			Enterobacteriaceae bacterium	DQ520801	99	0.0
	B 21	9	*Enterococcus* spp.	EU661816	99	0.0
		10	*Clostridium perfringens*	FM207095	99	0.0
		11	***Peptostreptococcus stomatis***	DQ160208	100	0.0
		12	*Clostridium perfringens*	FJ384379	99	0.0
	B 25	13	***Atopobium rimae***	AF292371	98	0.0
	B 26	14	***Bifidobacterium breve***	AF491836	99	0.0
		15	*Klebsiella* spp.	AB114636	96	2e − 34
		16	*Klebsiella* spp.	EF599758	99	0.0
			Citrobacter spp.	FJ587231	99	0.0
			Klebsiella oxytoca	FJ465169	99	0.0
			Citrobacter freundii	EU360794	99	0.0
		17	*Klebsiella* spp.	FJ615552	100	0.0
	B 27	18	***Bilophila wadsworthia***	AB117562	99	0.0
		19, 20	*Enterobacter cloacae*	FJ686827	99	0.0
			Enterobacter spp.	FJ976548	99	0.0
		21	*Clostridium perfringens*	FM207095	99	0.0
	B 28	22	*Atopobium rimae*	AF292371	98	0.0
		23	*Citrobacter freundi*	DQ517286	99	0.0
			Enterobacter cloacae	EF633997	99	0.0
			Klebsiella oxytoca	EU554427	99	0.0
			Klebsiella pneumoniae	EF633998	99	0.0
			Staphylococcus kloosii	DQ093351	99	0.0
		24	***Peptostreptococcus stomatis***	DQ160208	99	0.0
	B 29	25	*Enterobacter cloacae*	EU073021	99	0.0
			***Pantoea* spp.**	FJ560465	99	0.0
			Enterobacter hormaechei	FJ976588	99	0.0
			Enterobacter aerogenes	FJ976587	99	0.0
			Enterobacter spp.	GQ284539	99	0.0
		26	*Klebsiella* spp.	DQ267700	99	0.0
			Raoultella terrigena	EF634000	99	0.0
			Citrobacter freundii	FM207096	99	0.0
		27	*Clostridium perfringens*	AM889036	99	0.0
		28	***Gemella sanguinis***	NR_026419	99	0.0
	B 30	29	***Enterobacter cancerogenus***	FJ976582	98	5e − 47

Table 2. Continued.

Target region	Sample code	Band(s)*	Closest relative and GeneBank accession no.†		% Identity‡	*E* value
			Enterobacter spp.	GQ169799	98	5e – 47
			Enterobacter hormaechei	FJ976588	98	5e – 47
			Enterobacter aerogenes	FJ976587	98	5e – 47
			Pseudomonas putida	GQ330905	98	5e – 47
		30	*Enterobacter* spp.	FJ976548	100	0.0
	B 31	31, 32	*Clostridium perfringens*	FM207095	99	0.0
	B 32	33	***Atopobium rimae***	EU828366	99	0.0
		34	***Peptostreptococcus stomatis***	DQ160208	100	0.0
	B 33	35	*Escherichia coli*	GQ273519	99	0.0
			***Escherichia coli* O157:H7**	CP001368	99	0.0
		36	*Clostridium perfringens*	FM207095	99	7e – 180
		37	*Clostridium perfringens*	AM889036	99	0.0
28S rRNA gene	B 25	38	***Candida humilis***	FJ468459	100	2e – 46
		39	***Candida milleri***	U94923	98	4e – 19
			Kazachstania barnettii	FJ480849	98	4e – 19
			Candida humilis	GQ222344	98	4e – 19
	B 28	40	***Pichia guilliermondii***	AY497675	100	3e – 106
		41	***Pichia guilliermondii***	AY497675	99	1e – 100
			Candida carpophila	FM180531	99	1e – 100

*Bands are numbered on the basis of their position in the DGGE gels (data not shown).

†Accession number of the sequence of the closest relative found by BLAST search.

‡Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank DNA database.

The species identified by PCR-DGGE, but not grown in culture, are evidenced in bold.

Fig. 3. Macroscopical appearance of a segment cut from a clogged biliary stent (a) and of its yellow brownish sludge content (b) as results after bisecting the stent segment along its longitudinal axis.

Further, for the weak slime-producer strains of *C. difficile*, and *P. bivia* (Fig. 8) as well as for the two isolated strains of *C. fallax* (data not shown), it was possible to obtain a moderate development of a biofilm community after 48–72 h.

Discussion

A number of papers have reported possible hypotheses on the mechanisms presumably involved in the clogging phenomenon of biliary endoprostheses (for a review, see Donelli *et al.*, 2007).

To address the issue of how a biofilm could reach such a thickness to significantly narrow the lumen of the stent, it must be remembered that the biofilm exopolysaccharide matrix engulfs a number of 'foreign bodies' of different sizes including proteins, microbial byproducts, amorphous calcium bilirubinate and crystals of fatty acid calcium salts, as well as large-sized dietary fibers (Groen *et al.*, 1987; Leung *et al.*, 1988; Sung *et al.*, 1993;

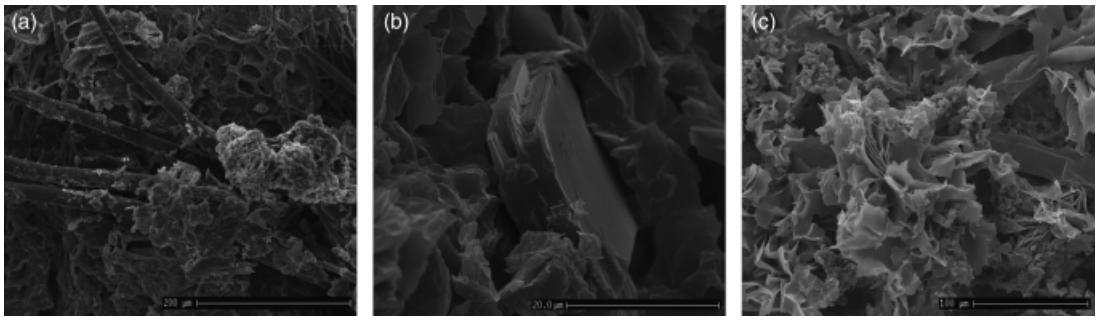

Fig. 4. Dietary fibers (a) and tentatively identified crystals of bile salts bilirubinate (b) and calcium palmitate (c).

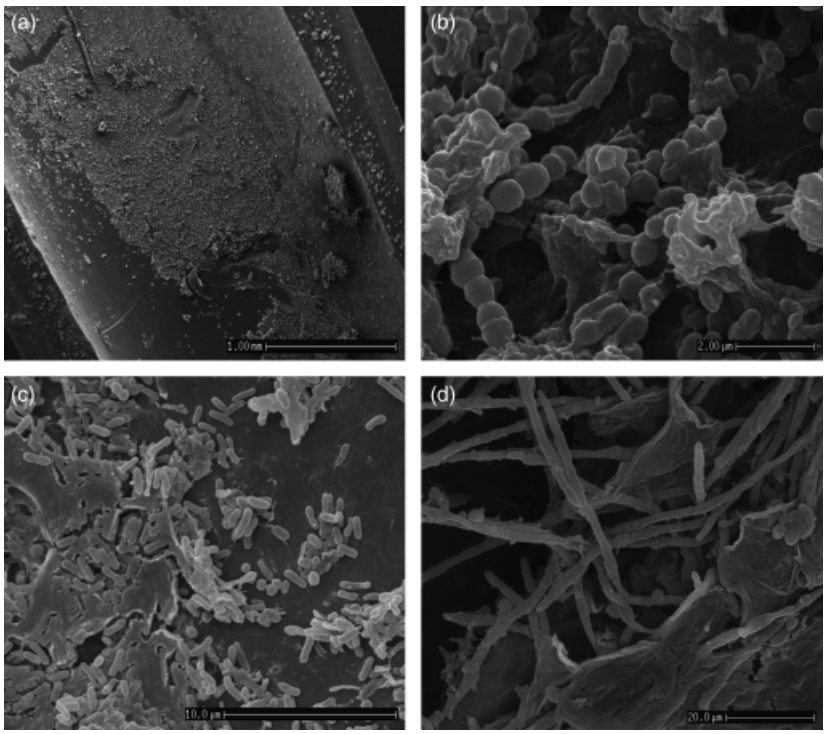

Fig. 5. SEM observation of the longitudinal section of a partially occluded stent (a), coccoid bacterial cells (b), rod-shaped bacteria (c) and fungal cells (d) observed at a higher magnification.

Basoli *et al*., 1999; Di Rosa *et al*., 1999; van Berkel *et al*., 2005).

Bile viscosity, which differs on the basis of a patient's health status, is another parameter to be considered. According to Poiseuille's law, if the bile viscosity increases, the maintenance of the same bile flow would require an increase in the inner stent diameter: it has been calculated that an increase of 0.2 mm in the inner stent diameter corresponds to a 300% increase in bile flow (Rey *et al*., 1985). In fact, the narrowing of the stent lumen, as a consequence of biofilm development, causes the slowing of bile flow, promoting both spontaneous and bacteria-driven bile salt precipitation. Thus, considering a mean bacterial diameter of about 1 μm, a reduction of 0.2 mm in a 10-Fr polyethylene stent (inner diameter 2.4 mm) would correspond to a biofilm of 200 overlapping bacterial layers. However, as already mentioned, the actual thickness of each bacterial layer is expected to be much higher because of the continuous engulfment of large-sized 'foreign bodies.' Further, the additional thickness of the host protein conditioning film, layered on the polymeric stent surface and known to mediate microbial attachment via specific adhesins, must be considered.

This model, based on the progressive reduction of the stent lumen as a consequence of the multispecies biofilm expected to develop in the peculiar luminal microenvironment of a biliary stent, can be considered, in our opinion, to be a reasonable way to approach the critical issue of stent clogging.

Moreover, the accumulation of biliary sludge is thought to be a multifactorial process in which, other than microbial

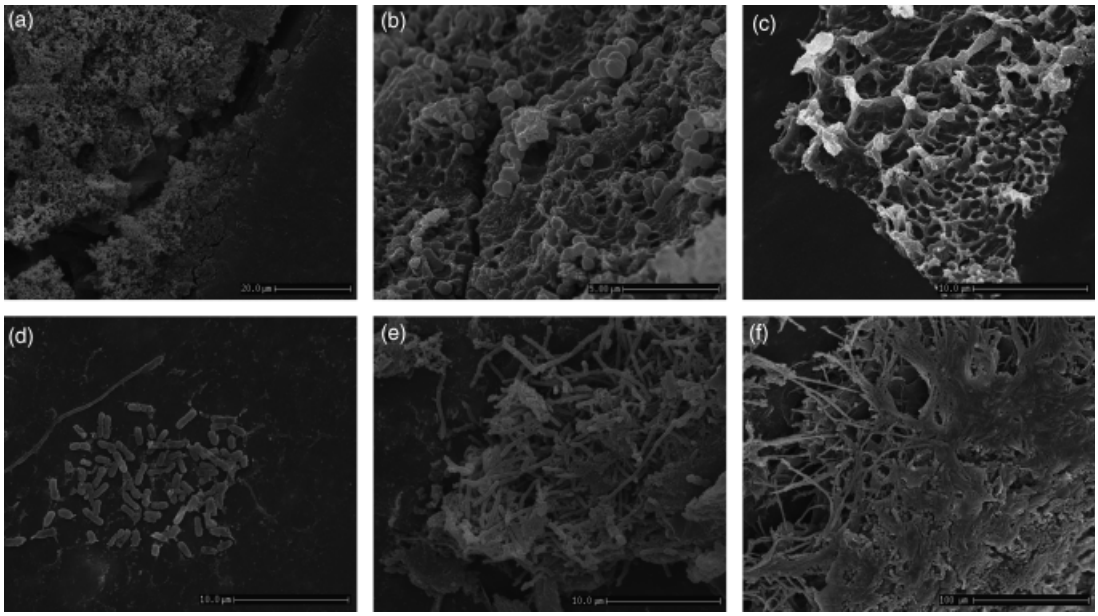

Fig. 6. SEM observation of a clogged biliary stent segment. Cross-section of a stent segment showing the cleavage between the sludge and the lumen occurring after sample dehydration (a); the appearance of the 'sludge side', still containing coccoid cells and their imprints (b) as well as of its counterpart devoid of bacteria, but still attached to the lumen surface (c) can also be observed. Clusters of microbial cells, closely bound to the polymeric surface of the stent lumen, are often detectable (d, e, f) in almost all the samples.

Table 3. Slime production by anaerobic strains isolated from occluded biliary stents

	Slime production
Gram-positive anaerobes	
Clostridium baratii	SP
Clostridium bifermentans	NP
Clostridium difficile	WP
Clostridium fallax	WP
Clostridium perfringens	SP
Peptostreptococcus magnus	SP
Gram-negative anaerobes	
Bacteroides capillosus	NP
Bacteroides distasonis	NP
Bacteroides fragilis	SP
Bacteroides oralis	NP
Fusobacterium necrophorum	SP
Prevotella bivia	WP
Prevotella intermedia	SP
Veillonella spp.	SP

SP, strong producers (OD > 0.240); WP, weak producers (0.120 < OD < 0.240); NP, nonproducers (OD < 0.120).

growth, slime production and biofilm formation, the activity of some bacterial enzymes is involved. It is known that β-glucuronidase, produced by *E. coli* and *Clostridium* spp. (Sakaguchi *et al.*, 1983; Leung *et al.*, 2001), deconjugates bilirubin, which combines with calcium ions, precipitating as calcium bilirubinate, thus increasing the amount of sludge (Maki *et al.*, 1984). In addition, phospholipase C, which is able to hydrolyze biliary lecithin causing the precipitation of calcium palmitate, has been evidenced in *Clostridium* spp. (Leung *et al.*, 2000).

According to the microbiological data obtained from this study, all except for one explanted biliary stent were colonized by a mixed microbial population. Isolates belonging to both aerobic and anaerobic bacteria as well as to fungi were identified. Among the aerobic bacteria, Gram-positive *Enterococcus faecalis* was the most frequently isolated species.

In a recent paper by our group, *E. faecalis* and *Enterococcus faecium* strains isolated from biliary stents have been investigated for the presence of genes encoding for aggregation substance and adhesive properties (Donelli *et al.*, 2004). Virulence genes encoding for aggregation substance have been detected by PCR, and the ability of clinical isolates to adhere to *in vitro* cultured cells and to produce biofilm has been assessed. This study indicated that the production of slime exhibited by most enterococcal isolates plays an important role in the colonization and subsequently in the occlusion of biliary stents, suggesting that aggregation substance could be implicated in the occlusion process and that enterococci carrying aggregation substance genes could have a selective advantage in endoprosthesis colonization as also reported by others (Waar *et al.*, 2002).

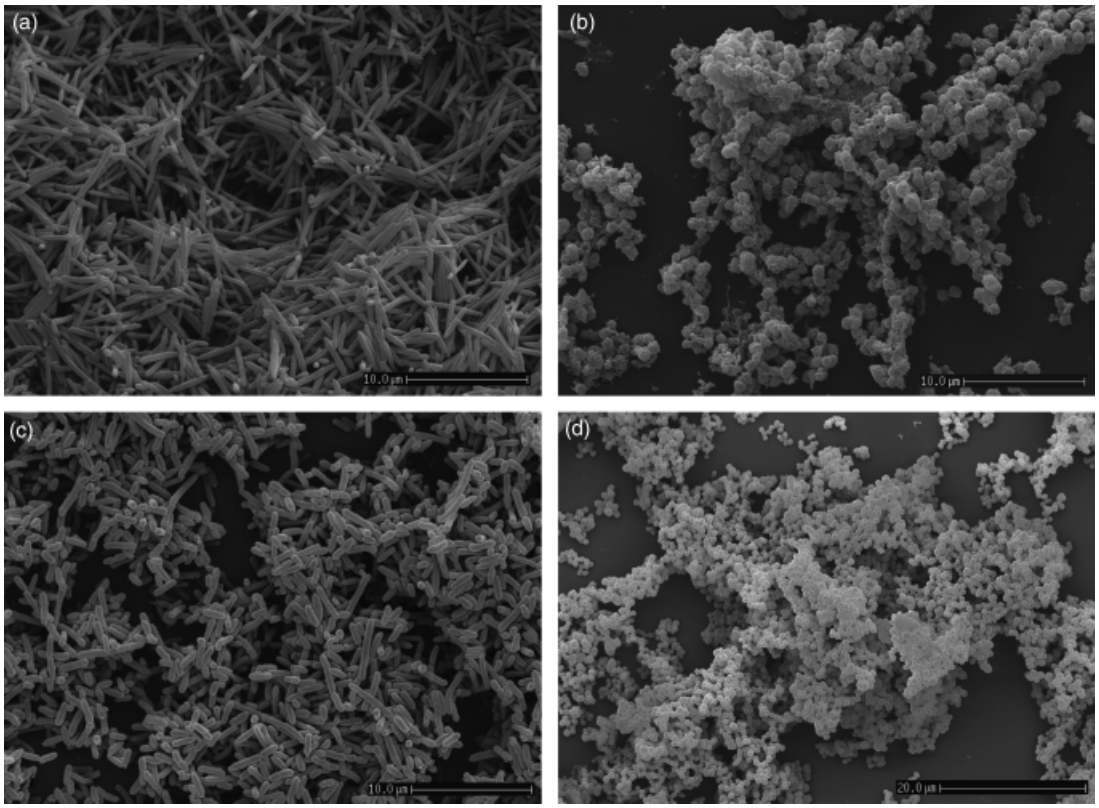

Fig. 7. SEM micrographs of biofilms developed, after a 24-h incubation, by anaerobic strains isolated from clogged biliary stents, belonging to the species *Fusobacterium necrophorum* (a), *Veillonella* spp. (b), *Bacteroides fragilis* (c) and *Peptostreptococcus magnus* (d).

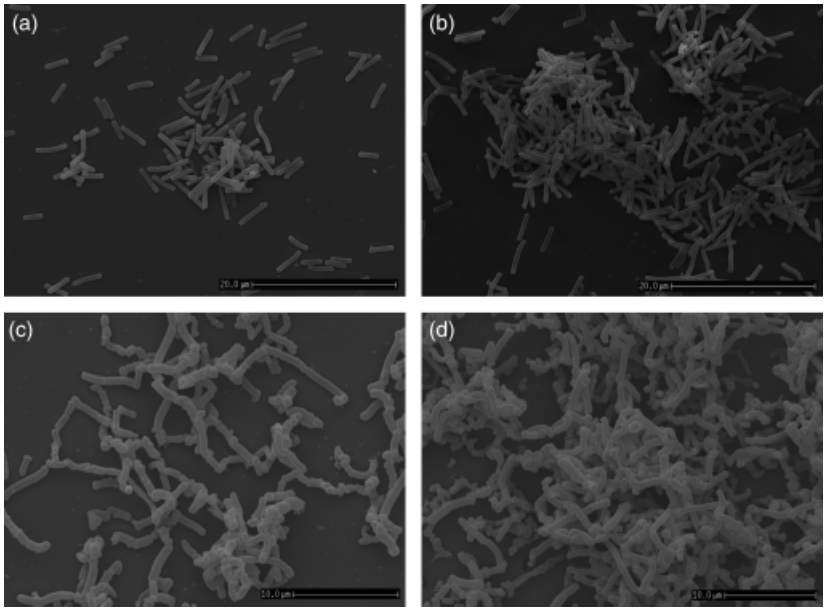

Fig. 8. SEM micrographs of biofilms developed, after a 24-h incubation (a, c) and a 48-h incubation (b, d), by the weak slime-producer anaerobic strains isolated from clogged biliary stents, belonging to the species *Clostridium difficile* (a, b) and *Prevotella bivia* (c, d).

Among Gram-negative bacteria, the most frequent aerobic species were *E. coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Enterobacter* spp., all of them well known as biofilm formers.

Bacteroides fragilis, *P. intermedia* and *Veillonella* spp. among Gram-negative bacteria and *Clostridium* spp. among Gram-positive bacteria were the most frequently isolated anaerobes that, in this study, were shown to be able to form a biofilm.

With respect to fungal strains, two were identified as *Candida albicans* and *Candida parapsilosis*, both well known as biofilm formers (Lattif *et al*., 2009; Ramage *et al*., 2009), the other six strains being identified only at the genus level.

The combination of cultivation procedures and DNA-based techniques (PCR-DGGE analysis) has led to an improved knowledge of the complex microbial community involved in the colonization of biliary stent lumen.

DGGE of PCR-amplified rRNA gene amplicons is a useful technique for monitoring the dynamic changes in a mixed bacterial population over time. The basis of this technique is that PCR-amplified DNA fragments of the same size, but differing in base pair sequences, which are specific for a given species, can be separated on a denaturing gradient gel performed using urea and formamide. DGGE allows the separation of these amplicons, producing a 'molecular fingerprinting' of the microbial species. This technique has been increasingly applied in environmental and food microbiology as well as in the analysis of microbial communities in the human body, including the intestinal tract (Donskey *et al*., 2003; Gafan *et al*., 2005).

In fact, the application of PCR-DGGE analysis to the biliary sludge occluding our stents allowed the identification of a large additional number of bacterial and fungal species that were not revealed by culture. The only partial overlapping between the species identified by PCR-DGGE and those isolated by culturing is presumably due to the different stent portions analyzed by both techniques as well as the PCR-DGGE analysis performed on only 50% of stents. In fact, the number of isolated species, as well as the ratio between aerobic and anaerobic species, may vary considerably depending on the portion analyzed. However, our findings of such a large number of anaerobic species, both isolated by culturing or identified by PCR-DGGE, can be considered of particular interest. Apart from the paper of Leung *et al*., (2000), which reported the isolation from unblocked biliary stents of strains belonging to only three anaerobic species (*C. perfringens*, *C. bifermentans* and *B. fragilis*), this is the first report on the isolation from blocked biliary stents of anaerobic strains belonging to 14 different species as well as on the identification of five additional species by PCR-DGGE.

Our SEM observations of sessile microorganisms remaining tightly attached to the surface of stent lumen after detachment of the covering amorphous material occurring during the dehydration process seem to significantly support the hypothesis that biliary stent clogging starts with the bacterial colonization of the stent lumen. This hypothesis finds a significant confirmation in the light micrograph of a cross-section of an occluded biliary stent recently published by Costerton (2007), in which concentric layers of a bacteria-rich biofilm are visible close to the inner surface of the stent lumen while large amounts of bile salts, mixed with dispersed small bacterial clusters, occupy the central part of the lumen, the remaining space allowing a slow bile flow.

The isolation of anaerobic bacteria in 57% of the analyzed stents and the demonstrated ability of the majority of them to form a biofilm *in vitro* strongly suggest that anaerobic species presumably play a significant role in biliary stent clogging. On the basis of these evidences and the well-known antibiotic tolerance of biofilm-growing bacteria, further studies should be focused on strategies to prevent biofilm development on the inner surfaces of biliary stents in order to prolong their patency with important medical and economical outcomes.

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