

1 **Bacterial invasion of the pancreas revealed after analyses of the pancreatic cyst fluids**

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16 Running head: Bacteria in Pancreatic cyst fluid

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5

6 **Abstract**

7 The involvement of bacterial translocation (BT) in the promotion of carcinogenesis has gained a  
8 considerable attention in the last years. At this point however BT has not been studied in the  
9 context of pancreatic cystic lesions and their development into pancreatic ductal adenocarcinoma.  
10 The aim of the study was to analyze if bacteria are present in pancreatic cyst fluid (PCF) collected  
11 from patients with suspected pancreatic cysts . Total DNA was isolated from sixty nine PCF. The  
12 occurrence of bacteria in PCF was analyzed using bacterial 16S rRNA gene-specific PCR-based  
13 method followed by sequence identification and quantitative PCR assay tuned up to different  
14 pathogenic and commensal human bacteria. Forty-seven out of sixty-nine samples (68%) were  
15 found positive for harboring bacterial 16S rRNA gene. Follow up sequencing analyses of the PCR  
16 products revealed that bacterial species related to *Fusobacterium spp.*, *Bacteroides spp.*, and  
17 *Bacillus spp.* were predominating the PCF samples. The results suggest that specific bacteria can  
18 translocate to the pancreas and become detectable in the PCF.

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## 1 Introduction

2 The incidence of pancreatic cystic lesions (PCL) in the general population is 2.4 percent.  
3 Neoplastic pancreatic cysts represent a risk for developing pancreatic ductal adenocarcinoma <sup>1</sup>  
4 (PDAC) and account for up to 5% of the total incidence of pancreatic cancerous lesions <sup>2,3</sup>. The  
5 clinical challenge in PCL is to identify signs of progressive neoplastic transformation in order to  
6 perform a surgical resection before a malignancy develops.

7 The vast majority of cysts nowadays are asymptomatic and coincidental finding at cross sectional  
8 imaging done for other reasons than cyst related symptoms. The percentage of cystic lesions being  
9 resected is increasing with the clinical intention of preventing the development of PDAC. Different  
10 types of cystic lesions are presently characterized by Grutzmann *et. al.*, and Farrell *et. al.*, which  
11 include the intra-ductal papillary mucinous neoplasm (IPMNs), mucinous cystic neoplasms  
12 (MCN), serous cyst adenomas (SCA), and pseudocysts <sup>4,5</sup>. IPMN's and MCN's pose a risk of  
13 developing into carcinoma of which IPMNs are being more prevalent compared with MCN's <sup>2,6</sup>.  
14 IPMNs are further classified as main branch, side branch or mixed types, based on the extent of  
15 involvement of the pancreatic ductal system <sup>5</sup>. Presently, there are no validated biomarkers to  
16 identify cystic lesions that require surgical resection. Although only up to three percent of the PCL  
17 patients would develop cystic lesions to malignancy, ten percent of the PCL patients are resected  
18 <sup>5</sup> suggesting the need for more superior clinical tests and better patients' stratification prior to  
19 surgery.

20 Currently, the decision for resection of PCL and/or continued monitoring are made  
21 according to the Sendai guidelines after evaluation of different clinical tests <sup>7-9</sup>. The available  
22 clinical tests include different biochemical analysis, cytology, pathological identification,  
23 endoscopic ultra sonography (EUS), and radiological diagnosis such as endoscopic retrograde  
24 cholangiopancreatography (ERCP), magnetic resonance cholangiopancreatography (MRCP) and  
25 whole body computerized tomography (CT). The inter-observer agreement, however, within and  
26 between different modalities remains moderate. Therefore, a set of preoperative biochemical  
27 analyses have been increasingly used in clinical decision making. This includes the study of cyst  
28 fluids and serum for the characteristic presence of carcinoembryonic antigen (CEA), cancer  
29 antigen 19.9 (CA-19.9), tumour associated glycoprotein 72-4 (CA-72-4), cancer antigen 15-3 (CA-  
30 15-3), pancreatic amylase, mucin antigens and along with cysts characteristics <sup>10,11</sup>. Other PCL

1 tests are based on specific analyses of different genetic modalities like K-RAS mutation and  
2 miRNA, but are still under investigation awaiting further clinical validation <sup>2,6,12,13</sup>. Though  
3 different diagnostic tests could help in decision making, none of them is sensitive and specific  
4 enough to make the right decisions for all patients involved. Additional markers are therefore are  
5 urgently needed to improve the management of PCL.

6 The human gut microbiome has emerged recently as an important environmental factor  
7 linked to the development of different intestinal and extra-intestinal malignancies <sup>14-16</sup>. Several  
8 members of the intestinal microbiome have been implicated in the bacterial translocation (BT) that  
9 could occur during different diseases of the pancreas <sup>17-21</sup>. Numerous studies suggest that BT takes  
10 place via the mesenteric lymph nodes route, followed by hepatic portal route, and trans mural or  
11 biliary or duodenopancreatic reflux <sup>18</sup>. This may initiate and/or accelerates intestinal leakage with  
12 a subsequent lowering of host immune response followed by dissemination of commensal gut  
13 microbiota and their by-products to other organs leading to sepsis and major multiple organ  
14 failures <sup>21,22</sup>. BT has been demonstrated in induced acute pancreatitis in mice and other animal  
15 models where BT takes place immediately towards the pancreatic duct causing necrosis in the  
16 exocrine system of pancreas <sup>19,23</sup>. Most of the BT has been described to originate from the small  
17 intestine rather than from the colon <sup>19</sup>. BT-associated intestinal pathobionts include different  
18 strains belonging to *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter fecalis*, *Proteus*  
19 *mirabilis*, and *Pseudomonas* species that have been involved in the necrotizing and acute  
20 pancreatitis <sup>18</sup>. Although there is ample evidence that BT takes place in various pancreatic diseases,  
21 not much has been studied nor understood about the bacterial presence in pancreatic cyst and/or  
22 cyst fluids. In this discovery phase study we have attempted to establish the bacterial occurrence  
23 in the pancreatic cystic lesions.

## 24 25 **Materials and Methods**

26 **Patient samples and Pancreatic cyst fluid collection.** A cohort of 103 patients with suspected  
27 cystic lesions was established between the period of 2008-2013 of which sixty nine samples were  
28 randomly selected for this discovery phase study (Table 1). The pancreatic cyst fluids (PCF) were  
29 collected after a signed informed consent from these patients who were undergoing endoscopic  
30 ultra sound fine needle aspiration (EUS-FNA) at the department of Gastroenterology &

1 Hepatology, Erasmus MC, The Netherlands. The PCF were collected, immediately transferred to  
2 the lab and stored at -150°C.

3 **DNA isolation.** Approximately 300 µl from sixty nine PCF samples were used for total DNA  
4 isolation. After bead beating (Fast Prep®-24 Instrument) the supernatant and pellet were separated  
5 by centrifugation at 13 000 rpm for 1 min and used for total DNA isolation using the Wizard DNA  
6 isolation kit as specified by the manufacturer's protocol (Catalogue no. A1620, Promega BNL  
7 B.V, The Netherlands). Isolated DNA was equilibrated in the DNA rehydration solution from the  
8 kit and quantified on nanodrop-2000 spectrophotometer (Isogen Life Science BV, De Meern, The  
9 Netherlands). PCF DNA was diluted to 1ng/µl for the PCR analyses and subsequently stored at -  
10 20°C.

11 **(q)PCR analyses.** Total DNA isolated from the PCFs were used for bacterial 16S RNA gene  
12 detection using both conventional PCR and qPCR. For the conventional PCR based method  
13 GoTaq® Flexi DNA polymerase kit was used (Promega BNL B.V, The Netherlands). qPCR was  
14 performed on IQ5 machine (Biorad, Bio-Rad Laboratories, Inc. Hercules, CA, United States of  
15 America) using Syber Green amplification kit (SYBR Select master mix, CFX, ABI, Life  
16 technologies, the Netherlands). All primers are listed in Table 2. All the primers were analysed  
17 and confirmed for the specificity to bacterial 16S rRNA and mismatch with the human  
18 mitochondrial 16S rRNA. 16S rRNA gene products from *Acinitobacteria spp.*, *Anaerococcus*  
19 *spp.*, *Bacillus spp.*, *Bacteroides spp.*, *F. nucleatum*, *Propionibacterium spp.* and *Staphylococcus*  
20 *spp.* were used to generate PCR standards from 10 ng to 0.00001 ng concentrations thereby  
21 achieving total of seven standards. Water was used as negative control.

22 **16S RNA gene sequencing analyses.** Classical Sanger sequencing method was done in order to  
23 identify the bacterial 16S rRNA genes present in the PCF. The PCR products generated from  
24 universal primers of 16S rRNA 16S bacterial gene were sequenced and then after identification of  
25 specific bacteria which are pathogenic commensals and as well involved in cancer development  
26 were used for further sequencing. The *Fusobacterium spp.*, *Bacteroides spp.*, *Anaerococcus spp.*,  
27 *Acinitobacteria spp.*, *Propionibacterium spp.*, and *Staphylococcus spp.* Specific 16S rRNA  
28 primers were used and sequenced (Table 2) through LGC Genomics GMBH, Germany.

29 **Fluorescent In-situ Hybridization.** The deparaffinization of the slides were performed as the  
30 protocol used in our lab <sup>24</sup>, once deparaffinized the tissues slides are subjected to PBS washing for

1 one minute. Modified proteinase K treatment was done under incubation at 45° C for 30 minutes  
2 to reduce the background staining (proteinase K – 20 µg/ml 1:10 dilution) (Sigma Aldrich, the  
3 Netherlands)<sup>25</sup>. The slides are then dehydrated for 3 min in 50, 70, and finally 96% ethanol. 500  
4 microliters of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5] 0.1% [wt/vol] sodium  
5 dodecyl sulfate) were applied and incubated at 37° C for 30 minutes. The hybridization buffer is  
6 washed off in the prewarmed PBS at 37° C and new pre-warmed hybridization buffer is added  
7 with the FITC labelled probes containing 1 picomole of FITC-labeled 16S rRNA probes (EUB338  
8 and *F. nucleatum*) was applied and incubated at 37° C for 18 hours. After hybridization, the slides  
9 were washed in 50 ml washing buffer (0.4X SSC buffer for 2 minutes followed with 2X SSC  
10 buffer for 1 minute, SSC buffer prepared as per the protocol by Zordan A et.al<sup>25</sup> and washed with  
11 PBS 1X for 1 minute and DAPI (1µg/ml) is added for counter staining of the nucleus and stained  
12 for exactly 3 minutes. The slides are washed with PBS 1X and air dried. All the staining are done  
13 in dark. The air dried slides are then examined using confocal microscope (LEICA, The  
14 Netherlands).

15 **Statistical methods.** All the statistical analysis were done using excel and Graphpad Prism 5.0.

16 The CT values in X axis were plotted against the serially diluted standards in Y axis to find  
17 the intercept Y using the exponential trend line for the CT values as per the excel formula. The R<sup>2</sup>  
18 is also determined for the same plot. The Y intercept is used to calculate the ng of 16S rRNA for  
19 the unknown samples of *Fusobacterium spp.*, *Bacteroides spp.*, *Anaerococcus spp.*,  
20 *Acinitobacteria spp.*, *Propionibacterium spp.*, and *Staphylococcus spp.*. Once the ng for each  
21 sample is obtained they are used for calculating absolute copy number of 16s rRNA using the  
22 following formula,

$$23 \quad 16S \text{ rRNA copy number} = \frac{(SX_1 * 6.02 \times 10^{14}) + PX_1 * 6.02 \times 10^{14}}{3.37 \times 10^2 * Q_1}$$

24 Where in SX<sub>1</sub> and PX<sub>1</sub> – S – stands for supernatant, P – stands for the pellet, X<sub>1</sub> – stands for the  
25 sample 1, 6.02 x 10<sup>14</sup> – nano gram to Daltons, 3.37 x 10<sup>2</sup> molecular weight of one nucleotide in  
26 Daltons, Q<sub>1</sub> – stands for the qPCR - product in number of nucleotides. The final conversion of  
27 16S rRNA copy number to 1ml of PCF is calculated via 16S rRNA copy number multiplied by  
28 3.33 (300 µl to 1ml) 300 µl of PCF fluid was used for total DNA isolation.  
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## 1 Results

2 **Study sample characteristics.** Sixty nine PCF samples were used for this study (Table 1). PCF  
3 were characterized as different types of IPMN's (39.1%), which included main branch IPMN  
4 (2.9%), mixed type IPMN's (5.8%), side branch IPMN's (17.4%) and multifocal side branch  
5 IPMN's (5.8%), and unclassified IPMN's (7.2%). Another types of PCF included in the study were  
6 mucinous cystic neoplasms (18.8%), pseudocysts (13.0%), serous cyst adenomas (13.0%). Finally,  
7 various other types of cystic lesions, gastrointestinal stromal tumour (GIST), neuroendocrine  
8 tumour (NET), and PCF without a definite clinical diagnosis were lumped into a separate category  
9 "other" PCF (15.9%) Table 1.

10 **Bacterial DNA presence in pancreatic cyst fluids.** We have found that 47 (68.11%) of the 69  
11 samples were positive for bacterial DNA originating from different bacteria (Table 3). Various  
12 PCFs harboured different percentage of positive samples ranging from 69.2% of all MCN's,  
13 followed by IPMN's with 66.7% and pseudocysts (66.7%), SCA's (55.6%), whereas in the group  
14 of others which included GIST, NET and clinically undefined samples were 71.4% predominantly  
15 positive for bacterial DNA (Table 6). This confirms the bacterial DNA present in the PCF's.

16 **Bacterial DNA quantification in pancreatic cyst fluids.** We have used qPCR experiments to  
17 confirm the presence of typical intestinal bacteria using specific primer sets targeting *Bacteroides*  
18 *spp.*, *Anaerococcus spp.*, *Acinitobacterium spp.*, *Propionibacterium spp.*, and selected pathobionts  
19 including *F. nucleatum*, and *Staphylococcus spp.* The copy number quantified for the total bacteria  
20 and the specific bacteria selected based on 16S rRNA qPCR were adjusted to ml of pancreatic cyst  
21 fluid are listed in Table 4. Based on the copy number the 16S rRNA were ranked the bacteria as  
22 represented in Table 5, which shows the pseudocysts ( $5.07 \times 10^8$ ) shows the highest copy number  
23 for bacteria and the lowest also seen in SCA ( $3.53 \times 10^5$ ), corroborating the presence of bacteria  
24 in pseudocysts as seen in earlier reports. The average bacterial 16S rRNA copy number /ml of PCF  
25 was  $1.08 \times 10^7$  with most occurring in pseudocysts ( $5.71 \times 10^7$ ) followed with MCN ( $1.15 \times 10^7$ ),  
26 IPMN ( $2.04 \times 10^6$ ) others (GIST, NET and clinically undefined –  $1.92 \times 10^6$ ), and SCA ( $7.91 \times$   
27  $10^5$ ) (Figure 1). One sample in pseudocysts, showed an abnormal bacterial load of  $5.07 \times 10^8$   
28 compared to other samples (Table 4). Similarly, there are change in the number of 16S rRNA copy  
29 number between various grades of dysplasia (graded according to the most atypical area in the  
30 lesion) in IPMN in the decreasing order:  $2.46 \times 10^6$  in no dysplasia, low grade dysplasia have  $1.75$



1  $\times 10^6$ , Moderate dysplasia  $9.67 \times 10^5$  and carcinoma in situ  $8.30 \times 10^5$  (Fig 2, no statistical  
2 difference). qPCR on PCF with mucinous cystic neoplasms have also demonstrated a higher  
3 number of bacterial 16S rRNA gene in the samples where no dysplasia is present (Fig 3).

4 When the partial gene bacterial 16S rRNA (1464 nucleotide) normal PCR were conducted  
5 using universal primers (table-2) only 68.11% of the samples showed positive bacterial population  
6 presence. Whereas the quantification via qPCR of bacterial 16S rRNA (193 nucleotides) primers  
7 shown in table-2, shows presence of bacterial 16S rRNA in 100% of the samples.

8 **Sequencing of the bacterial 16S rRNA sequences.** PCR products generated using universal 16S  
9 rRNA, and primers specific for *F. nucleatum*, *Bacteriodes spp.*, *Anerococcus spp.*,  
10 *Acinobacterium spp.*, *Propionibacterium spp.*, and *Staphylococcus spp.* were subjected to  
11 sequencing to confirm the qPCR results (Table 6). The sequencing results have demonstrated that  
12 *F. nucleatum* is present in 13 out of all 69 PCF samples (18.84%). Other predominating bacterium  
13 involved in PCF was *Bacillus spp.* which was present in 16 out of 69 (23.19%) samples. The  
14 presence of other bacteria was also noted which included *Ruminococcus spp.*, *Staphylococcus spp.*,  
15 *Caldimonas spp.*, *Arthrobacter spp.*, *Acinetobacter spp.*, *Bacteroides spp.*, *Orpinomyces spp.*  
16 *Anaerococcus spp* (combined data from Tables 3 and 6).

17 **Fluorescent In-situ Hybridization (FISH).** FISH was used as an additional analysis to confirm  
18 the presence of bacteria in PCF. Selected slides were blindly analysed checked and scored for  
19 bacterial presence by two personal specialized in confocal microscopy. We have confirmed it by  
20 the confocal imaging of the FISH slides for the bacterial presence Figure 4. Importantly, our  
21 control samples, negative for bacterial presence in the PCF's were also negative in the tissue slices  
22 generated after resection (data not shown). On the opposite side, the tissue samples from patients  
23 with PCF positive for bacterial DNA were also positive for bacterial presence in the tissue, the  
24 cysts borders and the duct borders (Figure 4).

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## 26 Discussion

27 In the current study we have demonstrated that 68 percent of the PCF harbour different  
28 types of bacteria. The possible BT into the pancreatic cysts involve also bacteria related to *F.*  
29 *nucleatum* implicated in adenoma to carcinoma transformation in epithelial cells<sup>26</sup>. Although



1 EUS-FNA is not a sterile procedure, 1/3 (32 percent) of the PCF samples were negative for  
2 bacterial DNA on our cultivation-independent bacteriological findings. Nonetheless EUS-FNA  
3 limitations for bacterial analyses need to be discussed. Before sampling cyst fluid, the needle tip  
4 is exposed to the content in the stomach or gut lumen before being punctured through the gut wall  
5 into the pancreatic cyst. The presence of bacterial 16S rRNA in all the 69 samples taken for this  
6 study, observed as in the qPCR is due to the above said reasons of EUS-FNA being a non-sterile  
7 procedure and also due to the bacterial products transferred via the other contaminants of the cyst  
8 fluids, like blood which is plausibly carrying the bacteria DNA via the bacteria ingested  
9 macrophages and other immune cells. To nullify and further account for that when interpreting our  
10 data, we have performed confocal imaging for bacteria confirming that the tissue samples resected  
11 from patients with PCF positive for bacterial DNA were also positive for bacterial presence in the  
12 tissue. Furthermore, many bacteria in the stomach like *H. pylori* and streptococci were not found  
13 in our PCF analyses supporting the notion that specific bacterial community may be associated  
14 with PCF.

15 BT involvement into the pancreatic abscess, necrosis, pancreatitis, and pancreatic cyst have  
16 also been reported mainly in isolated case studies, but never in large cohort analyses <sup>22,27-29</sup>.  
17 Numerous case studies identified BT to pancreas were caused by the commensal bacteria and  
18 fungi. Pancreatic infections mainly arise from translocation of bacteria from the small bowel, and  
19 rarely from the colon and oropharyngeal route as demonstrated by study on *Veillonella* and  
20 *Bifidobacterium spp.* which were identified in pancreatic abscess <sup>28</sup>. A study of Brook et al., has  
21 identified 158 bacterial species from pancreatic abscess of which 77 isolates were aerobic and rest  
22 81 were anaerobic bacteria <sup>30</sup>. Most commonly detected microorganisms in pancreatic pseudocysts  
23 include often not only opportunistic bacteria like *E. coli*, *Enterobacter spp.*, *Klebsiella spp.*, and  
24 *Staphylococcus spp.*, but also fungal isolates including *Candida albicans* (15 case studies) <sup>27</sup>.  
25 Importantly, EUS FNAB procedure caused serious *Clostridium perfringens* infections in 5 patients  
26 leading to pancreatitis and pancreatic cyst formation, which required surgical interventions <sup>31</sup>. The  
27 study has illustrated the nature of the bacterial transfer from the early to mid-gut commensal  
28 bacteria to the pancreas. Yet none of these earlier studies have directly proved the presence of  
29 bacteria in the pancreatic cysts and its fluid. In the current study we have demonstrated that  
30 bacterial DNA is present in PCF. Although we do not have direct evidence that bacteria are alive  
31 in the PCF samples, the successful FISH analyses for some of the samples suggest that at least in

1 part the bacterial population comprises of intact bacteria with undegraded 16S rRNA. PCF and the  
2 pancreatic duct borders represent therefore a niche that may become colonized by specific bacteria  
3 as demonstrated in the study.

4 The interactions between commensal and/or pathogenic bacteria and their metabolic  
5 products with hosts tissue can affect several diseases' progression <sup>27,29,32</sup>. Human serum derived  
6 antibody response against oral bacteria *Porphyromonas gingivalis* ATTC 53978 has been  
7 associated with an increased risk of pancreatic cancer by two folds <sup>33</sup>. Furthermore, a study of  
8 Farell et.al., has showed that two oral bacteria, *Neisseria elongata* and *Streptococcus mitis*, can  
9 differentiate between pancreatic cancer and normal cases with 96.4% sensitivity and 82.1%  
10 specificity <sup>35</sup>. In the same study, the oral bacteria *Granulicatella adiacens* and *Streptococcus mitis*  
11 differentiated between pancreatic cancer and pancreatitis with 85.7% sensitivity and 55.6%  
12 specificity, similarly in colorectal cancer the difference in bacterial Operational Taxonomic Units  
13 (OTU's) were found between the normal, adenomatous and cancerous population, resulting in  
14 diagnosis of these three types in a person <sup>34-36</sup>. Analyses on the direct role of *Helicobacter pylori*  
15 have shown that the bacterium was not associated with the development of pancreatic cancer <sup>37-40</sup>.  
16 Two other studies, however, support indirect association between *H. pylori* and pancreatic cancer  
17 risk. *H. pylori* may represent high risk for the individuals with non-O blood types because  
18 *Helicobacter spp* DNA was found in 75% patients with pancreatic adenocarcinoma and in 57% of  
19 patients with neuroendocrine cancer, and in 60% of patients with chronic pancreatitis <sup>41,42</sup>. Also  
20 the recent establishment of the role of *F. nucleatum*, *Bacteriodes spp.* in colorectal cancers in *in-*  
21 *vivo* and *Citrobacter rodentium*, in in-vitro conditions also shows the association of bacteria in  
22 general in cancer initiation and development <sup>26,33,36,43-46</sup>. Microbiomes roles is being established in  
23 other cancers like urothelial (*Bacteriodes spp.*) <sup>47</sup> and prostate cancers (*Propionibacterium spp*)  
24 <sup>48,49</sup>, liver cancer where the gut microbial metabolites were involved in the liver cancer progression  
25 <sup>50</sup>.

26 Through this study we conclude, the bacterial presence was confirmed in the pancreatic cyst fluids,  
27 and as well-established via their copy number of 16S rRNA, Sangers sequencing and fluorescent  
28 in-situ hybridization. The kind of bacterial 16S rRNA shown, prove they were translocated from  
29 the mid and hind gut into the pancreas. Further analyses of the type of bacteria present in PCF

1 can potentially help us to distinguish between various cyst types and the difference between the  
2 various grades of dysplasia.

3 It may also help in understanding the mechanism, whether it is a epiphenomenon or direct effect  
4 of how the presence of specific type of bacteria can potentiate the primary cystic lesions  
5 progressing towards pancreatic cancer. Furthermore, the data also raise a question of whether the  
6 bacterial population as seen in the pancreatic cyst fluid as well the cyst margins and duct margins  
7 could be a possible commensals of the pancreatic duct rather than bacterial invasion/translocation.  
8 A more comprehensive study is required for understanding this phenomenon.

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## Bacterial 16S rRNA (EUB338 probe) in-situ hybridization in resected tissue

DAPI









