**PANCREATITIS**

Detection and identification of bacterial DNA in serum from patients with acute pancreatitis

E de Madaria, J Martinez, B Lozano, L Sempere, S Benlloch, J Such, F Uceda, R Francés, M Pérez-Mateo

**Background and aims:** Bacterial infections are common complications in patients with acute pancreatitis, and translocation of bacteria from the intestinal lumen is probably the first step in the pathogenesis of these infections. As blood cultures in afebrile patients are usually negative, more sensitive methods to investigate this hypothesis in patients are needed. Our group has recently developed a method to detect the presence of bacterial DNA in biological fluids, and we aimed to detect bacterial DNA in patients with acute pancreatitis, as molecular evidences of bacterial translocation. 

**Methods:** Samples of blood were obtained on three consecutive days within the first six days after admission. Bacterial DNA was detected using a polymerase chain reaction based method, and an automated DNA nucleotide sequencing process allowed identification of bacteria species. 

**Results:** Thirty one consecutively admitted patients with acute pancreatitis were studied. Bacterial DNA was detected in six patients (19.3%), and the sequencing process allowed identification of *Citrobacter freundii* and *Pseudomonas aeruginosa*. In two patients the same bacteria detected at admission was detected 24 hours later (above 99.9% homology of nucleotide sequence). Basic clinical and biochemical characteristics were similar among patients with or without the presence of bacterial DNA. 

**Conclusion:** Detection of gram negative bacteria derived bacterial DNA in our series supports the contention that bacterial translocation is a systemic process in approximately 20% of patients with acute pancreatitis that does not seem to be related to the severity of the episode or immediate development of infection.

**Patients and Methods**

AP was defined as the presence of compatible abdominal pain together with an increase in plasma amylase three times above the upper limit of normality, and abnormal pancreatic morphology on image techniques. The severity of the episode was determined according to the Atlanta criteria. 

**Abbreviations:** AP, acute pancreatitis; BT, bacterial translocation; GNB, gram negative bacteria; bactDNA, bacterial DNA; PCR, polymerase chain reaction; CRP, C reactive protein; bp, base pairs

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and the presence or absence of pancreatic necrosis. Pancreatic necrosis was defined as focal or diffuse well marginated zones of non-enhanced pancreatic parenchyma that were larger than 3 cm or involved more than 30% of the area of the pancreas.\textsuperscript{23} C reactive protein (CRP) was measured 48 hours from admission in all patients, and all local and systemic complications were recorded.

Blood from each patient was obtained for routine haematological, biochemical, and coagulation studies. Blood was inoculated at the bedside in aerobic and anaerobic blood culture bottles, 10 ml each, and in rubber sealed pyrogen-free tubes of 5 ml each (Endo Tube ET; Chromogenix AB, Vienna, Austria). Subsequent blood samples were collected under aseptic conditions every 24 hours over a three day consecutive period within the first six days after hospital admission and inoculated in rubber sealed pyrogen-free tubes (Endo Tube ET; Chromogenix AB).

### DNA isolation

DNA extraction and PCR amplification of the complete 16S ribosomal RNA gene were performed in all serum samples using the method described previously by our group.\textsuperscript{22}

After blood extraction, specimens were processed in airflow chambers and tubes were never exposed to free air. All laboratory procedures were performed by the same investigator (BL). Serum was obtained by centrifuging blood at 3500 rpm for 10 minutes. DNA was extracted with a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) from 200 μl of serum incubated in AL buffer with proteinase K for 30 minutes at 56°C, and applied onto QIAmp Spin Columns. DNA was finally eluted with 50 μl of 70°C preheated AE buffer. The yield and purity of DNA were measured by reading A\textsubscript{260} and A\textsubscript{260}/A\textsubscript{280} in a BioPhotometer (Eppendorf).

The sensitivity, specificity, and limit of detection of the method have been determined previously,\textsuperscript{22} with an absolute limit of bactDNA detection of 10 pg/ml.

### DNA amplification

PCR reactions for the complete amplification of the 16S ribosomal RNA gene were carried out. Universal primers were: 5’-AGA GTT TGA TCA TGG CTC AG-3’ as forward (located at positions 8–27) and 5’-GTT TAC CTT GGT ACG ACT T-3’ as reverse (positions 1509–1491).\textsuperscript{24} Approximately 10–100 ng of template were added into a reaction mix containing 20 mM Tris HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 μl of each deoxynucleoside triphosphate, 0.4 μM of each primer, and 2.5 U Taq DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, California, USA) to complete a final volume of 50 μl. To avoid false positive results, positive and negative controls were performed in duplicate in each assay. DNA from Escherichia coli was added as a positive control, and sterile water and PCR mixtures (without template) were used as negative controls. PCR was carried out in a Mastercycler personal (Eppendorf) through the cycles as follows: an initial cycle of 95°C for four minutes was followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, with a final extension period at 72°C for 10 minutes to complete the cycling sequence. Total PCR reaction volume was filtered through QIAquick Spin Columns (QIAquick PCR Purification Kit; Qiagen) to remove primers and nucleotides.

Purified products (5 μl) were visualised on 1.5 % agarose gels stained with ethidium bromide. A band of approximately 1500 base pairs (bp) was obtained, corresponding to the specific amplification of the prokaryotic 16S ribosomal RNA gene.

### DNA sequencing

PCR amplicons were partially sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) and ABI PRISM 310 automated sequencer according to the manufacturer’s indications. The same forward primer used for PCR amplification was used for sequencing approximately 600 bp. Obtained sequences were compared with 16S rRNA sequences available both in the Ribosomal Database Project and the GenBank and EMBL obtained from the National Center for Biotechnology Information Database by the advanced BLAST search (www.ncbi.nlm.nih.gov).

### Statistical analysis

CRP data are reported as median (25th and 75th percentiles). Statistical differences were analysed using the χ\textsuperscript{2} test for categorical data applying the Yates’ correction when required or the Mann-Whitney U test for quantitative data. All p values were two tailed. A p value <0.05 indicated statistical significance. Analysis was performed with the SPSS statistical package (SPSS Inc. v 10.0; Chicago, Illinois, USA).

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
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<tr>
<td>8</td>
<td></td>
<td></td>
<td>C freundii*</td>
<td>C freundii*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
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<td>C freundii</td>
<td>ND</td>
<td></td>
<td></td>
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<td>15</td>
<td>P aeruginosa*</td>
<td>ND*</td>
<td></td>
<td>ND*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>C freundii*</td>
<td>ND*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Filled areas represent days on which blood was obtained for culture and bacterial DNA detection (with bacteria detected indicated). ND, no detection of bacterial DNA.

Blank areas represent days on which no blood samples were collected.

*Samples obtained after the beginning of antibiotic prophylaxis.
RESULTS
From January 2003 to November 2003, 31 consecutively admitted patients with AP were included in this study. Thirteen patients were male, with a mean age 57 (SD 20) years. The aetiology of AP was biliary in 14 cases, alcoholic in six, related to hypertriglyceridaemia in one patient, idiopathic in eight cases, and due to other causes in two patients. The clinical course of AP was mild in 22 patients and severe in the remaining nine cases. Complications related to the episode were one case of aseptic pancreatic necrosis, one case of renal impairment, one respiratory insufficiency, one case of shock, and four pancreatic pseudocysts. Three infections were detected, including one respiratory, one urinary, and one on a pancreatic necrosis, in different patients.

All patients with AP of biliary origin underwent surgery on admission, except for one patient who developed peripancreatic collections progressing to a pancreatic pseudocyst. One patient with infected pancreatic necrosis was treated with imipenem and then underwent early surgery (necrosectomy and set in place of draining tubes), with a good clinical outcome. After surgery only one patient required vasoactive drugs because of hypotension refractory to general insufficiency, one case of shock, and four pancreatic pseudocysts. Three infections were detected, including one respiratory, one urinary, and one on a pancreatic necrosis, in different patients.

Infections developed during hospitalisation and its relationship to bactDNA detection and identification

Table 2 shows all infections that developed in patients with AP (table 1), being mild in four and severe in two cases. Staphylococcus epidermidis was isolated in the only positive blood culture in this series (patient No 29), which we considered was due to skin contamination of the culture, and this patient was not included in the evaluation of patients with translocation. Three of six patients with bactDNA received prophylactic intravenous imipenem (500 mg three times daily) on admittance to the emergency unit. The aetiology of AP was considered idiopathic in three patients, biliary in two, and ischaemic in one case. All PCR fragments were sequenced for bacterial identification. Clinical and microbiological characteristics of the patients in whom BT was detected as well as the results of bacterial DNA identification are detailed in table 2. Citrobacter freundii was detected in patient Nos 6, 8, 12, and 20, and Pseudomonas aeruginosa in patient Nos 15 and 18. Similarity of the isolated bactDNA sequence present in the database was in most cases more than 99%, which is high enough to warrant identification of the species. BactDNA corresponded to the same bacteria in two consecutive samples obtained from patient Nos 8 and 15 (table 1). The similarity of nucleotide sequences isolated in the consecutive samples in both patients was greater than 99.9%.

Detection and identification of bacterial DNA
BactDNA was detected in eight samples obtained from six patients with AP (table 1), being mild in four and severe in two cases. Staphylococcus epidermidis was isolated in the only positive blood culture in this series (patient No 29), which we considered was due to skin contamination of the culture, and this patient was not included in the evaluation of patients with translocation. Three of six patients with bactDNA received prophylactic intravenous imipenem (500 mg three times daily) on admittance to the emergency unit. The aetiology of AP was considered idiopathic in three patients, biliary in two, and ischaemic in one case. All PCR fragments were sequenced for bacterial identification. Clinical and microbiological characteristics of the patients in whom BT was detected as well as the results of bacterial DNA identification are detailed in table 2. Citrobacter freundii was detected in patient Nos 6, 8, 12, and 20, and Pseudomonas aeruginosa in patient Nos 15 and 18. Similarity of the isolated bactDNA sequence present in the database was in most cases more than 99%, which is high enough to warrant identification of the species. BactDNA corresponded to the same bacteria in two consecutive samples obtained from patient Nos 8 and 15 (table 1). The similarity of nucleotide sequences isolated in the consecutive samples in both patients was greater than 99.9%.

In this study, we detected bactDNA in blood of a subgroup of patients with AP, thus providing molecular evidence of BT. Our results suggest that BT is a systemic process.

DISCUSSION
In this study, we detected bactDNA in blood of a subgroup of patients with AP, thus providing molecular evidence of BT. Our results suggest that BT is a systemic process.
that changes in the intestinal flora may induce impairment of the intestinal barrier, as shown by the presence of an abnormal intestinal permeability in this setting. Moreover, most of the bacteria commonly responsible for bacterial infections in patients with AP are Enterobacteriaceae of intestinal origin, and it has been shown that GNB infections in patients with AP are preceded by intestinal colonization of the same bacteria species. From a different point of view, the risk of developing a septic complication is higher in patients with AP and colonic overgrowth by GNB, and this is associated with increased mortality. It is likely that changes in the intestinal flora may induce impairment of the intestinal barrier and induction of associated BT.

Studies of the presence of bactDNA in biological fluids in patients with AP and other pathologies have rarely been reported in the literature. To our knowledge, only one study investigated the presence of bactDNA in blood in a series of patients with AP. Similar to our investigation, blood was obtained at three different time points during the first week of admission, and the authors failed to detect bactDNA in any patient. This is surprising given the simultaneous presence of positive blood cultures in some of the patients. This conflicting result may be due to different methodological problems, such as the level of sensitivity of the PCR or the search for bactDNA in whole blood, as it has been reported that both haemoglobin and lactoferrin may inhibit PCR.

As stated in the methods section (see positive control DNA and negative controls) in our investigations all bactDNA negative samples were inoculated with a known amount of bactDNA and tested again to rule out the hypothetical presence of inhibitors of PCR that could induce false negative results.

Our group recently described detection of bactDNA in serum and ascitic fluid from a series of patients with decompensated cirrhosis, and in a subsequent study the same authors showed that the presence of bactDNA in serum persisted for as long as 72 hours in a different subset of patients with identical clinical characteristics. The authors considered that persistence of bactDNA in blood was probably the consequence of repeated episodes of BT and not of the predominance of a reduced clearance of bacterial fragments. Interestingly, in both studies detection of bactDNA was not associated with more advanced liver disease or short term development of infection by the same bacteria species.

In the present investigation we detected bactDNA in six of 31 patients with AP. Although it has been reported that BT would be more frequent in patients with severe AP, we did not find a significant relationship between detection of bactDNA and severity of the process, presence of pancreatic necrosis, or associated radiological findings. In only two patients with bactDNA in blood was AP considered severe, according to actual criteria. As the severity of the episode of AP is the consequence of the clinical evolution, and we do not know what mechanisms are intimately related to the presence in blood of bactDNA in this setting, it is difficult to explain the relationship between BT and the severity of the episode of AP using the present study design.

BactDNA is constituted by short repeated sequences of unmethylated CpG dinucleotides, known as CpG motifs. Different experimental studies have demonstrated that these fragments are capable of inducing a similar immune response to that produced by a complete microorganism in vitro, thus becoming potent activators of cells of the innate immune system through joining Toll-like receptor 9. Our group has also shown that bactDNA induces potent macrophagic activation with liberation of proinflammatory cytokines and this could, in turn, increase the bactericidal activity of the media, making immediate bacterial colonisation less likely. Taken together, we may hypothesise that BT develops in some patients with AP irrespective of the severity of the process, and that infection may take place in cases were a sufficient number of viable bacteria find the adequate substrate, such as in those with pancreatic necrosis or in patients with decreased activity of the immune system.

Interestingly, CRP levels in patients with bactDNA showed a tendency to be higher than those in patients without bactDNA. CRP is an acute phase protein that has been shown to have prognostic utility in patients with AP. Increased levels of CRP in patients with bactDNA may be a result of the immune response to the presence of bactDNA or, conversely, one of the causes inducing translocation.

In our series, three of six patients with bactDNA were receiving antibiotic prophylaxis with imipenem. This may be explained by the fact that intravenous imipenem is not excreted in bile and affects only slightly the intestinal flora. Furthermore, as our method detects not only viable but also non-viable bacteria in blood, the translocating bacteria, probably killed by plasma levels of antibiotics, may be equally detectable.

The only patient with an infected pancreatic necrosis was due to E coli, when bactDNA from P aeruginosa was detected at admission. This apparent discrepancy may be explained by the time elapsed between bactDNA detection and development of the infection (24 days). It is not known if BT due to a certain bacterial species at a certain time point may be followed by BT due to a different bacterial species along the inflammatory process. In fact, abnormal intestinal permeability in patients with AP has been described previously by our group and others.

C freundii was identified in patient Nos 6, 8, 12, and 20. Although to our knowledge there is no information in the literature regarding the pathogenic role of C freundii in the development of infections in patients with AP, this bacteria has been shown to translocate in different experimental and clinical situations and it is related to both biliary and intra-abdominal infections. As bactDNA detection and identification in patients with AP is a new approach to the study of BT in humans, we do not know if C freundii is a frequent translocating agent in patients with AP.

In conclusion, detection of GNB derived bactDNA in our series supports the contention that BT is a systemic process in approximately 20% of patients with AP that does not seem to be related to the severity of the episode or associated with an immediate development of infection. More information is needed to ascertain the mechanisms leading to BT and its clinical and immunological consequences in the short and long term in patients with AP.

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Conflict of interest: None declared.

REFERENCES


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