Identification of Bacterial DNA in Noninfectious Pleural Fluid with a Highly Sensitive PCR Method

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Key Words

Bacterial translocation • Genomics • Pleural effusion

Abstract

Background: Bacterial DNA due to bacterial translocation has been identified in noninfectious ascitic fluid samples.

Objective: This study investigated the possible presence of bacterial DNA in the pleural fluid of patients with pleural effusions of noninfectious origin, using a highly sensitive PCR-based method. **Methods:** Pleural fluid samples from 175 patients (average age $\pm$ SD: 69 $\pm$ 14 years) with noninfectious pleural effusion (62 transudates, 113 exudates) were analyzed. Bacterial DNA was detected using nested PCR with amplification of a fragment of the gene r16S, with 2 amplification protocols, i.e. low sensitivity (10 and 40 cycles) and high sensitivity (40 and 40 cycles). **Results:** With the less sensitive amplification process, only 1 sample was positive (*Haemophilus parainfluenzae* in a patient with hepatic hydrothorax). With the highly sensitive nested PCR method, bacterial DNA was identified in the pleural fluid, of both transudative and exudative origin, of 75 of the 175 patients (43%). In cases of isolation of a single bacterium, the more frequent were *Escherichia coli*, *Salmonella enterica* and *Streptococcus pneu-*

moniae. **Conclusions:** Regardless of its origin, bacterial DNA can be identified in almost half of noninfectious pleural effusions by using a highly sensitive PCR-based method. The possible clinical significance or prognostic value of these findings deserves to be evaluated.

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Introduction

Modern molecular methods in microbiology can identify the presence of minimal amounts of bacterial DNA in clinical samples that are not detected by conventional culture approaches [1, 2]. Interactions between bacteria and the host represent a continuum from transient contact through colonization to pathogenic activity and ultimately infection [1]. Consequently, the fact that bacterial DNA is found at normally sterile sites may not necessarily imply the presence of an infection, and this finding may be due to bacterial translocation. Bacterial translocation is defined as the passage of bacteria generally from the gastrointestinal tract to other sites, such as lymph nodes, liver, spleen, kidney and bloodstream [3, 4]. The three mechanisms classically considered to promote bac-

Table 1. Etiology of pleural effusions and PCR results

	Number	PCR positive	PCR negative
Transudates	62	30 (48%)	32 (52%)
Hepatic hydrothorax	22	14 (64%)	8 (36%)
Cardiac failure	38	15 (39%)	23 (61%)
Urinothorax	1	1	0
Duropleural fistula	1	0	1
Exudates	113	45 (40%)	68 (60%)
Malignant	69	30 (43%)	39 (57%)
Idiopathic	26	9 (35%)	17 (65%)
Pulmonary embolism	5	1 (20%)	4 (80%)
Postsurgery	4	2	2
Post-traumatism	3	1	2
Dermatomyositis	1	1	0
Pleuropericarditis	1	1	0
Rheumatoid arthritis	2	0	2
Hemothorax	1	0	1
Renal failure	1	0	1
Total	175	75 (43%)	100 (57%)

Results of nested PCR with 40 cycles/40 cycles (see Patients and Methods).

terial translocation are intestinal bacterial overgrowth, increased permeability of the intestinal mucosal barrier and deficiencies in host immune defenses [4]. However, different conditions have been associated with bacterial translocation, including operations, cardiopulmonary bypass, thermal injury, smoke inhalation, heat stroke or rigid bronchoscopy [5–7].

Several studies published in recent years have detected and identified bacterial DNA in about one third of non-infectious ascitic fluid samples in patients with cirrhosis, and this has been considered to be due to episodes of bacterial translocation and systemic seeding [7–14]. In fact, experimental studies in rats have confirmed that the presence of bacterial DNA in fluids is a marker of bacterial translocation [15].

It is reasonable to presume that bacterial DNA due to translocation might also be detected in pleural fluid, at least in cases of hepatic hydrothorax. In addition, circumstances that favor bacterial translocation may also be present in other clinical situations in patients with pleural effusion. For these reasons, we investigated the possible presence of bacterial DNA in the pleural fluid of patients with pleural effusions of noninfective origins, using a highly sensitive PCR-based method.

Patients and Methods

Patients

This study was approved by the Clinical Research Ethics Committee of the Hospital General Universitario, Elche, Spain (01/10/07). Pleural fluid was obtained from diagnostic thoracentesis of 200 consecutive patients. Part of each pleural fluid sample was analyzed bacteriologically (aerobic and anaerobic cultures and Lowenstein culture), and standard biochemical and cytological studies were also performed. A fraction of the remaining sample was stored at -20°C for subsequent PCR determination. Twenty-five patients were excluded because an infectious disease was established as the cause of the effusion (21 parapneumonic pleural effusions, 3 due to tuberculosis, 1 due to actinomycosis). Of the patients with noninfectious pleural effusions included in the study ($n = 175$), 105 were male (60%) and 70 female (40%). The average age ($\pm$ SD) was 69 ± 14 years. The diagnoses were established by the attending physicians, who were not aware of the results of the molecular microbiological study. The etiologies of the pleural effusions are shown in table 1.

Molecular Microbiology Studies

DNA Extraction

DNA extraction was performed using the QIAamp[®] DNA Mini Kit (Qiagen, Hilden, Germany) system after cell lysis with lysozyme of the sediment obtained after centrifuging 10 ml of pleural liquid at 4,000 rpm (2,250 g) for 20 min.

Bacterial DNA Detection using Nested PCR

A fragment of the gene r16S was amplified using the primers 27F (5'-agagtttgatcmtggctcag-3') and 1492R (5'-gytacctgttacgacctt-3'). Using the amplicon as target, another amplification was carried out with the primers 357F (5'-cctacgggaggcagcag-3') and 1221R (5'-cattgtagcagtagcc-3') [16]. Figure 1 shows a diagram of the process. Both reactions took place in the thermocycler Gen-Amp[®] PCR System 9700 (Applied Biosystems, Foster City, Calif., USA). In the first reaction, samples were amplified at 94°C for 20 min, 50°C for 20 min and 72°C for 3 min, and in the second at 94°C for 20 min, 51°C for 20 min and 72°C for 3 min. If in the second amplification process an amplification was detected in 2% agarose gel by the presence of a fragment of 864 bp, this was sequenced by MacroGen (Seoul, Korea).

Two amplification processes were performed, one with 10 cycles in the first amplification and 40 cycles in the second (low-sensitivity process), and the other, a high-sensitivity process, with 40 cycles in each amplification (with this methodology it is possible to detect the presence of fewer than 10 copies of bacterial DNA in the sample) [17]. The level of sensitivity of this method was established accordingly with previous studies on *Mycobacterium tuberculosis* [17], with the extraction of DNA from dilution series of *Streptococcus pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* previously quantified by culture (Nanodrop, Wilmington, Del., USA). In all the reactions, positive and negative controls were included and the positive samples were studied again with different reactants so as to rule out the possibility of cross-contamination of the amplicants.

The obtained sequences were compared with sequences in the GenBank database. In case of lack of homology, the program RipSeq (Isentio, Bergen, Norway) was used. This method is able to determine different species in the case of mixed bacterial DNA.

Fig. 1. Schematic representation of nested PCR with amplification of the target gene RNAr 16S.

Statistical Analysis

Data are presented as numbers and percentages. The frequency of positive results in transudates and exudates was compared using the χ^2 test. A p value lower than 0.05 was considered statistically significant.

Results

In all cases, the cultures in a conventional medium were negative for aerobic and anaerobic bacteria and mycobacteria. With the less sensitive amplification process (10 cycles/40 cycles), there was only 1 positive result in a patient with hepatic hydrothorax, identified as *Haemophilus parainfluenzae*. With the highly sensitive nested PCR method (40 cycles/40 cycles), bacterial DNA was identified in the pleural fluid of 75 of the 175 patients (43%). Bacterial DNA was detected in pleural effusions of both transudates and exudates of different origins. The etiology of the pleural effusions with positive and negative results is detailed in table 1. The difference in the proportion of positive cases in transudates and exudates was not statistically significant ($p = 0.27$).

In cases of detection of a single bacterium, the more frequent were *E. coli*, *Salmonella enterica* and *S. pneumoniae*. The microorganisms identified are listed in table 2. In 38 of the 75 cases, bacterial DNA contained more than a single microorganism. In these cases, a web-based

application designed for the analysis of mixed DNA was used (RipSeq, Isentio), and bacteria (1 or more) were identified in 17 cases (table 3).

Discussion

The identification of bacterial DNA at a sterile site, such as pleural fluid, is used to establish the microbiological etiology of a pleural infection [18]. However, the fact that bacterial DNA is found does not necessarily mean that there is an infection [1]. Bacteria may be detected at various sites as a result of colonization or translocation. Subsequent development of infection depends on various possible factors, such as the number and viability of the bacteria, the virulence and pathogenicity of the organism and the ability of the host to mount an effective immune response [1].

In this study, bacterial DNA was detected and identified in 43% of the pleural fluid samples using a highly sensitive detection system. The negative results obtained with conventional cultures and PCR techniques with fewer amplifications show that the amount of bacterial DNA was minimal and thus, logically, not due to an infectious process but to bacterial translocation. This study was performed using an extremely sensitive methodology intended to detect the presence of just a few DNA mol-

Table 2. Microbiological results

Identified microorganisms	Number	Etiology of pleural effusion
Mixed	38	hepatic hydrothorax (n = 7), cardiac failure (n = 7), urinothorax (n = 1), malignant (n = 16), idiopathic (n = 4), pulmonary embolism (n = 1), postsurgery (n = 1), dermatomyositis (n = 1)
<i>Escherichia coli</i>	7	hepatic hydrothorax (n = 1), cardiac failure (n = 2), malignant (n = 2), idiopathic (n = 2)
<i>Salmonella enterica</i>	6	hepatic hydrothorax (n = 2), malignant (n = 3), idiopathic (n = 1)
<i>Streptococcus pneumoniae</i>	6	hepatic hydrothorax (n = 1), cardiac failure (n = 3), malignant (n = 1), pleuropericarditis (n = 1)
<i>Streptococcus</i> sp.	2	hepatic hydrothorax, malignant
<i>Salmonella</i> sp.	2	malignant (n = 2)
<i>Enterobacter</i> sp.	2	malignant, postsurgery
<i>Klebsiella oxytoca</i>	2	malignant, idiopathic
<i>Kocuria</i> sp.	1	hepatic hydrothorax
<i>Haemophilus parainfluenzae</i>	1	hepatic hydrothorax
<i>Streptococcus mitis</i>	1	malignant
<i>Pantoea agglomerans</i>	1	cardiac failure
<i>Moraxella</i> sp.	1	cardiac failure
<i>Escherichia</i> sp.	1	cardiac failure
<i>Pseudomonas</i> sp.	1	malignant
<i>Fusobacterium nucleatum</i>	1	malignant
<i>Pseudomonas reactans</i>	1	idiopathic
<i>Staphylococcus aureus</i>	1	post-traumatic

Table 3. Microbiological identification in cases of mixed bacterial DNA

Identified microorganisms	Etiology of pleural effusion
<i>Staphylococcus</i> sp. ¹	hepatic hydrothorax (n = 2), malignant (n = 1)
<i>Salmonella</i> sp., <i>Enterobacter</i> sp., <i>Citrobacter</i> sp.	hepatic hydrothorax
<i>Citrobacter</i> sp., <i>Cronobacter</i> sp., <i>Sighella</i> sp.	hepatic hydrothorax
<i>Propionibacterium</i> sp. ¹	malignant (n = 1), cardiac failure (n = 1)
<i>Bacillus</i> sp., <i>Propionibacterium</i> sp.	malignant
<i>Anaerococcus</i> sp., <i>Shigella</i> sp., <i>Escherichia coli</i>	malignant
<i>Citrobacter</i> sp., <i>Cronobacter</i> sp.	malignant
<i>Pantoea agglomerans</i> , <i>Enterobacter</i> sp.	malignant
<i>Clostridium</i> sp., <i>Haematobacter</i> sp.	cardiac failure
<i>Streptococcus</i> sp., <i>Acinetobacter</i> sp., <i>Propionibacterium</i> sp.	idiopathic
<i>Propionibacterium</i> sp., <i>Klebsiella oxytoca</i>	idiopathic
<i>Acinetobacter</i> sp., <i>Enterobacter</i> sp., <i>Propionibacterium</i> sp.	pulmonary embolism
<i>Sphingomonas paucimobilis</i> , <i>Paracoccus</i>	postsurgery
No dominant bacterial sequences identified	22 remaining cases

¹ Two species.

ecules in each sample, as opposed to the methodologies used in the clinical diagnosis of infections in pleural fluid, whose usefulness and efficacy are well known [18–20].

Following the publication of various papers describing bacterial translocation in ascitic fluid [8–14], we decided to investigate the possibility of detecting the presence of bacterial DNA in pleural fluid. Very small amounts of bacterial DNA may not be detected by conventional PCR methods; therefore, we used a highly sensitive method of nested PCR with 40 amplifications in each of the amplification processes (40 cycles/40 cycles). In addition, we also performed the study with fewer amplifications (10 cycles/40 cycles) in order to determine whether this bacterial DNA could also be detected by less sensitive methods. In this case, a positive result would imply that there was a greater amount of DNA. Not surprisingly in view of the results in ascitic fluid, we found bacterial DNA in as many as 43% of pleural samples, both transudates and exudates, of a wide variety of etiologies using the highly sensitive nested PCR method. Only 1 sample gave a positive result with the less sensitive method (10 cycles/40 cycles). The negative results of the less sensitive amplification process and conventional cultures suggest that the bacteria are present in very small quantities and may not be viable, since, in the bacterial translocation process, the bacteria pass from the intestine to the mesenteric lymph nodes and may be altered by the immune system due to phagocytosis by polymorphonuclear leukocytes [21]. Since our method detects bacterial DNA rather than bacterial cells, it is possible that our amplifications correspond to circulating naked DNA.

Our data suggest that the bacterial DNA detected may have originated in the intestinal mucosa (since enterobacteria were found) or pulmonary mucosa (*Streptococcus* spp.). These findings may be explained by the decrease in antibacterial defenses, as well as classical mechanisms of bacterial translocation [22]. The mucosae, both of the lung and intestine, are lined by a monolayer of ep-

ithelia which provides a tissue barrier and transport function. A strong association between bacterial translocation and mucosal inflammatory disease has been demonstrated [23].

This translocation process is highly conditioned by various immunological mechanisms, the characteristics of some pathogens, use of antibiotics or even mechanical factors in the airways, as demonstrated in experimental pneumonia [1, 24, 25].

The significance of the identification of bacterial DNA in noninfectious pleural effusions may be an interesting factor to analyze in future prospective studies in view of the data obtained with ascitic fluid. In patients with cirrhosis, bacterial translocation appears to occur in a subgroup of patients with advanced disease [11] and is associated with the activation of the cell-mediated immune response [15, 26, 27] and the complement system [28]. It seems that the presence of bacterial DNA in serum and ascitic fluid is an independent factor in noninfected patients with the cirrhosis and implies a poor prognosis [15, 29]. Recent studies suggest that a genetic variant associated with impaired mucosal barrier function may also be involved in this process [30].

To conclude, we identified bacterial DNA in 43% of noninfectious pleural fluid samples using a highly sensitive PCR-based method. The effusions in which bacterial DNA was detected were of different origins, including transudates and exudates and benign and malignant diseases. The possible clinical significance or prognostic value of these findings deserves to be evaluated.

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