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FEMS Microbiology Ecology 40 (2002) 107–112

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases

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Received 4 September 2001; received in revised form 22 January 2002; accepted 23 January 2002

First published online 12 March 2002

Abstract

We have searched for sulfate-reducing bacteria in the feces of 41 healthy individuals and 110 patients from a Hepato-Gastro-Enterology Unit using a specific liquid medium (Test-kit Labège®, Compagnie Française de Géothermie, Orléans, France). The 110 patients were separated in 22 patients presenting with inflammatory bowel diseases and 88 patients hospitalized for other lower ($n = 30$) or upper ($n = 58$) digestive tract diseases. Sulfate-reducing bacteria were isolated from 10 healthy individuals (24%), 15 patients presenting with inflammatory bowel diseases (68%), and 33 patients with other symptoms (37%). A multiplex PCR was devised for the identification of *Desulfovibrio piger* (formerly *Desulfomonas pigra*), *Desulfovibrio fairfieldensis* and *Desulfovibrio desulfuricans*, and applied to the above isolates. The strains of sulfate-reducing bacteria consisted of *D. piger* (39 isolates), *D. fairfieldensis* (19 isolates) and *D. desulfuricans* (one isolate). The prevalence of *D. piger* was significantly higher in inflammatory bowel disease patients (55%) as compared to healthy individuals (12%) or patients with other symptoms (25%) ($P < 0.05$). © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Sulfate-reducing bacterium; Inflammatory bowel disease; *Desulfovibrio*

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) of unknown etiology, but they are likely to rely on environmental, genetic and immune factors [1]. An infectious origin has been proposed, and many microorganisms have been implicated in the absence of any convincing arguments. However, in both syndromes, the intestinal inflammation does respond to antibiotherapy. In animal models of chronic colitis, luminal flora is an essential cofactor for the disease to occur [2,3]. This may explain the renewed interest in the role of the bowel flora as a cause of these disorders [4–6].

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that conduct dissimilatory sulfate reduction to obtain energy, resulting in the release of a great quantity of sulfide. They are commonly isolated from environmental sources, but are also present in the digestive tract of animals and humans. As *Desulfomonas pigra* has been reclassified as *Desulfovibrio piger* comb. nov. [7], human isolates of SRB consist almost exclusively of *Desulfovibrio* species [8–11]. Recent findings suggest that SRB may have a role in human diseases. They have been associated with the clinical severity of human periodontitis [12], and isolated from profound abscesses (abdominal or brain), blood or urine [13–16]. In these settings, most strains have been identified as *Desulfovibrio fairfieldensis*, a recently proposed new species [16], by 16S ribosomal RNA gene (16S rDNA) sequencing. *Desulfovibrio desulfuricans*, the type species of the genus *Desulfovibrio*, has also been isolated from human specimens [14]. The implication of SRB in IBD has been suggested as their metabolic end product, hydrogen sulfide, is a cytotoxic compound [9,17–19]. This compound may act through an inhibition of butyrate oxidation, the main energy source for colonocytes. The impairment of the functions of the intestinal

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PII: S0168-6496(02)00201-5

epithelium would lead to cell death and chronic inflammation. However, the species of SRB associated with IBD have not yet been identified. Their identification would permit to look for virulence factors as well as their susceptibility to antimicrobial agents.

In medical laboratories, SRB are seldom isolated from human samples because of a slow growth. Colonies appear after more than 3 days of incubation and are not noticed, being overgrown by the accompanying flora, unless they are the dominant or sole species present. Thus, their search in feces is difficult unless a specific medium is used. Such media usually contain an organic compound (electron donor and carbon source), sulfate (electron acceptor), iron and a reducing agent. The growth of SRB in specific culture media is easily detected by a blackening of the medium due to hydrogen sulfide (H_2S) production resulting in the formation of a ferrous sulfide precipitate. Once isolated from clinical samples, identification at the species level may be difficult. For example, it is not possible to differentiate *D. fairfieldensis* and *D. desulfuricans* by phenotypic tests. Thus, gene amplification is a valuable tool to achieve such an identification.

The major aim of this study was to determine which species of *Desulfovibrio* may be associated with IBD if any. For this purpose, a specific liquid medium (Test-kit Labège®, Compagnie Française de Géothermie, Orléans, France) was used for the growth of the SRB from the feces of healthy individuals and of patients hospitalized in the Hepato-Gastro-Enterology Unit of the Centre Hospitalier et Universitaire de Nancy, France. A multiplex PCR was devised for the identification of the isolates at the species level.

2. Materials and methods

2.1. Patients

Feces of 41 healthy individuals (17 men and 24 women; mean age 38 years, range 1–101 years) and 110 patients (67 men and 43 women; mean age 57 years, range 14–100 years) from the Hepato-Gastro-Enterology Unit of the Centre Hospitalier et Universitaire de Nancy, France, were collected. Healthy individuals consulted consecutively for a checkup. No pathogenic microorganism was found in their feces. Healthy individuals and patients have not had any antibiotic administration in the month before the sample was obtained. Patients were separated into three groups: IBD (n = 22) included 17 CD and five UC; other lower bowel diseases (n = 30) included 23 patients presenting with mild or moderate symptoms such as abdominal pain, intestinal transit troubles and rectorrhagia, and seven patients with colonic cancer; upper digestive tract diseases (n = 58) included gall stones, cirrhosis, hepato-cellular carcinoma, gastric and pancreatic tumors. IBD were diagnosed on clinical, endoscopic and histological findings. Most of the IBD patients presented with an active disease (Table 1).

2.2. SRB detection and enumeration

One gram of feces was mixed with 4 ml of phosphate buffered saline buffer and centrifuged (3000 rpm, 5 min). One milliliter of supernatant was inoculated immediately in a liquid medium (Test-kit Labège®, Compagnie Française de Géothermie, Orléans, France) according to the manufacturer's instructions. Briefly, the Test-kits Labège® consist of vials containing 9 ml of a specific medium (organic compounds: lactate and acetate, reducing agent: titanium citrate) anaerobically conditioned for SRB detection. It is inoculated with a syringe through a rubber cap. This limpid and colorless medium has been originally devised for the detection of SRB from environmental samples. It was compared to the commonly used Postgate's solid medium E (organic compound: lactate, reducing agents: ascorbic acid and thioglycolic acid) [20], inoculated in parallel under anaerobic atmosphere. SRB were enumerated using long and narrow tubes filled up with the latter medium and inoculated with decimal dilutions of the feces. All inoculated media were incubated at 37°C for 2 months. The presence of SRB was ascertained by the formation of a black precipitate (ferrous sulfide) in liquid media and by the appearance of black colonies in solid media.

2.3. Design of PCR primers

The 16S rDNA sequences of *Desulfomonas* and *Desulfovibrio* strains available in the GenBank database were compared using the Sequence Navigator software, version 1.0.1 (Applied Biosystems Inc., Foster City, CA, USA). It permitted to design six primers for the identification by

Table 1
Characteristics of patients with IBD

IBD	Age (years)		Sex		Stage of the disease[a]		Patients with anti-inflammatory and/or immunosuppressive agents
	Mean	Range	Female	Male	Active	Remission	
Crohn's disease (n = 17)	40	18–74	8	9	11	6	13
Ulcerative colitis (n = 5)	39	14–78	2	3	3	2	4

[a]Activity of IBD was evaluated on clinical, endoscopic and histological findings.

Table 2
Primers for the identification of *Desulfovibrio* strains

Strains	Primers	Length of the PCR product (bp)
D. piger	Pig-F, P687-R	255
D. desulfuricans Essex 6	Essex-F, P687-R	255
D. desulfuricans MB	27K-F, 27K-R	396
D. fairfieldensis	Fair-F, P687-R	534

PCR of the SRB previously isolated from humans [7,10,13–16], related respectively to *D. piger* (formerly *Desulfomonas pigra*) ATCC 29098^T, *D. desulfuricans* Essex 6 ATCC 29577^T, *D. desulfuricans* MB ATCC 27774, and *D. fairfieldensis* ATCC 700045. *D. desulfuricans* Essex 6 and *D. desulfuricans* MB were differentiated as the 16S rDNA sequences of these strains exhibit a difference of 3% [14]. The primers were 27K-F (5′-CTG CCT TTG ATA CTG CTT AG-3′), 27K-R (5′-GGG CAC CCT CTC GTT TCG GAG A-3′), Essex-F (5′-CTA CGT TGT GCT AAT CAG CAG CGT AC-3′), Fair-F (5′-TGA ATG AAC TTT TAG GGG AAA GAC-3′), Pig-F (5′-CTA GGG TGT TCT AAT CAT CAT CCT AC-3′), and P687-R (5′-GAT ATC TAC GGA TTT CAC TCC TAC ACC-3′) (Table 2). The specificity of these primers was checked on all bacterial sequences available from the GenBank database using the Blast program, version 2.0 (National Center for Biotechnology Information, Bethesda, MD, USA).

2.4. SRB identification by multiplex PCR

Four collection strains (*D. piger* ATCC 29098^T, *D. desulfuricans* Essex 6 ATCC 29577^T, *D. desulfuricans* MB ATCC 27774, and *D. fairfieldensis* ATCC 700045) and 12 clinical strains (two strains related to *D. desulfuricans* Essex 6, two strains related to *D. desulfuricans* MB and eight strains identified as *D. fairfieldensis*) were used as positive controls. The sensitivity of the PCR was evaluated with dilutions of quantified bacterial strain suspensions. The specificity of the PCR was checked with negative controls including type strains (*Bilophila wadsworthia* ATCC 49260^T, *Desulfovibrio gigas* DSM 1382^T, *Desulfovibrio vulgaris* DSM 644^T) and common intestinal clinical strains belonging to the following species: *Bacteroides fragilis*, *Bacteroides merdae*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides vulgatus*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium innocuum*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Eubacterium exiguum*, *Eubacterium lentum*, *Fusobacterium nucleatum*, *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morganii*, *Peptostreptococcus magnus*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus bovis*.

At the end of incubation time, all the 151 inoculated Test-kits Labège® were checked using the multiplex PCR. DNA extracts were obtained from 500 μl of culture media, after centrifugation and resuspension in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). Briefly, the cells were lysed using successively lysozyme (3 mg ml^{-1}), SDS (1%, w/v) and proteinase K (0.25 mg ml^{-1}). After an overnight incubation at 37°C, DNA was extracted by the standard phenol/chloroform/isoamyl alcohol method. Each 50-μl PCR mixture contained 5 μl of DNA extract (approximately 50 ng of DNA) and final amounts of 0.4 μM of each primer, 0.8 mM of each deoxynucleoside triphosphate (Boehringer Mannheim Biochemicals, Mannheim, Germany), 0.4 mM of Tris–HCl buffer, 1.5 mM $MgCl_2$, and 1.5 U of *Taq* DNA polymerase (Gibco BRL Life Technologies, Paisley, UK). All reactions were carried out using the GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA). An initial denaturation step of 94°C for 4 min was followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min), and with a final extension (72°C, 5 min). Negative (water instead of DNA extract) and positive (*D. fairfieldensis* DNA extract) controls were included in each run. Amplified products were resolved by electrophoresis in 1.5% (w/v) agarose gels containing ethidium bromide (1.6 mg ml^{-1}). A 100-bp DNA ladder was used as a size marker (Gibco BRL Life Technologies, Rockville, MD, USA). *D. piger*, *D. desulfuricans* Essex 6, *D. desulfuricans* MB and *D. fairfieldensis* were identified by a 255-, 255-, 396- and 534-bp band, respectively. *D. piger* and *D. desulfuricans* Essex 6 were further differentiated by separate PCR assays using their respective specific primers. For negative samples, 16S rDNA amplification using the consensus primers 27f and 1525r [16] was performed to check the absence of inhibition of the PCRs by contaminants.

3. Results

3.1. SRB detection and enumeration

In healthy individuals and according to the above criteria, SRB were found in 10 feces (24%) with both Test-kit Labège® and Postgate's medium. In patients from the Hepato-Gastro-Enterology Unit, SRB were grown from 42 feces using Postgate's medium. Three additional samples were found positive with the Test-kit Labège®. Thus, among the 110 patients studied, SRB were detected by culture in 45 patients (41%). Three additional samples gave equivocal results with the Test-kit Labège® (presence of a dark brownish precipitate). The mean times of detection of the growth of SRB were 2 and 6 days using the Test-kit Labège® (range 1–11 days) and Postgate's medium (range 3–39 days), respectively. The mean count of SRB in the feces of healthy individuals and patients was 10^5 g^{-1} (range 10^2–10^9 g^{-1}). Thus, SRB count was not related to the clinical status of patients.

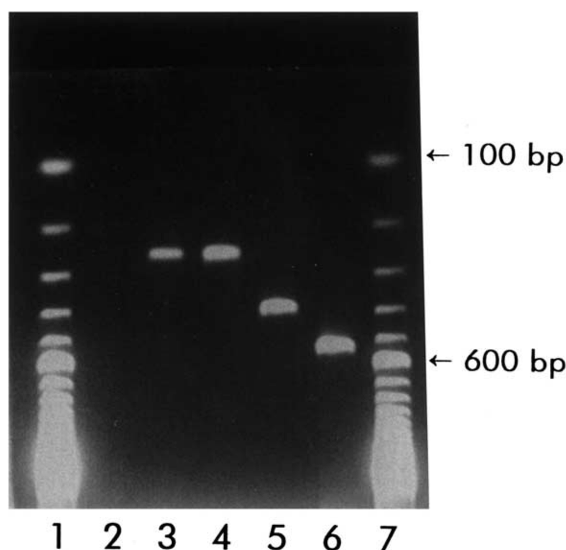

Fig. 1. Multiplex PCR products obtained with four different strains of *Desulfovibrio*. Lanes: 1 and 7, 100-bp DNA ladder; 2, negative control (water); 3, *D. piger* (formerly *Desulfomonas pigra*) ATCC 29098^T; 4, *D. desulfuricans* Essex 6 ATCC 29577^T; 5, *D. desulfuricans* MB ATCC 27774; 6, *D. fairfieldensis* ATCC 700045.

3.2. SRB identification by multiplex PCR

The sensitivity of the multiplex PCR assay was 100 bacteria per ml. Its specificity was ascertained by the absence of cross-reactions between the four genospecies differentiated. Each positive control was found positive solely with the corresponding set of primers. All strains used as negative controls to check the specificity, including the type strains of *D. gigas* and *D. vulgaris*, gave negative results with the four primer sets. The specificity of the reactions was further confirmed by sequencing the amplified products. The obtained sequences always corresponded to the ones expected. For SRB-negative samples by PCR, 16S rDNA amplification permitted to discard the possibility of an inhibition of the PCRs by contaminants.

In healthy individuals, the 10 positive feces corresponded to *D. piger* ($n = 5$), *D. fairfieldensis* ($n = 4$) or *D. desulfuricans* MB ($n = 1$) (Fig. 1). Each culture-positive feces was found positive by PCR for only one of the genospecies tested.

In patients from the Hepato-Gastro-Enterology Unit, the 45 positive and the three equivocal feces gave positive results by PCR. They corresponded to *D. piger* ($n = 33$), *D. fairfieldensis* ($n = 14$) or both ($n = 1$). No *D. desulfuricans* was evidenced (Table 3). The culture-negative flasks were also negative by PCR.

3.3. Relation of Desulfovibrio species with IBD

The distribution of the species did not differ significantly when comparing healthy individuals and patients with non-inflammatory bowel diseases. *D. piger* was barely more prevalent than *D. fairfieldensis*. Conversely, in patients with IBD, *D. piger* was 4 times more frequent than *D. fairfieldensis*. This difference was especially noticeable for CD as IBD patients consisted mainly of CD patients. Furthermore, the prevalence of *D. piger* was significantly higher in patients hospitalized for IBD as compared to healthy individuals or patients hospitalized for other pathologies ($P < 0.05$). There was no relation between the stage of the disease and the presence of SRB. Therapy did not modify the isolation rate of these bacteria.

4. Discussion

The presence of SRB in the intestinal tract of animals and humans has been recognized for a long time, although identifications at the species level have seldom been performed. Our results confirm that these bacteria are common inhabitants of the intestinal tract of humans. Most studies of intestinal SRB from IBD patients have relied on cultivation-based microbiological analysis of fecal samples, and have therefore identified SRB at the genus level [8,9,11]. However, recent studies have suggested that the possible role of SRB in the pathogenesis of IBD may be related to physiological and/or phylogenetic differences between strains of SRB [21,22]. Thus, we devised a multiplex PCR to identify these bacteria at the species level. Considering the difficulty of isolation and the rarity of specific

Table 3
Identification of SRB in feces by PCR according to the clinical status of patients

Clinical status	SRB[a]	*D. piger*[b]	*D. fairfieldensis*	*D. desulfuricans*
Inflammatory bowel diseases ($n = 22$)	15 (68)	12 (55)	3 (14)	0 (0)
Crohn's disease ($n = 17$)	12 (71)	11 (65)	1 (6)	0 (0)
Ulcerative colitis ($n = 5$)	3 (60)	1 (20)	2 (40)	0 (0)
Non-inflammatory bowel diseases ($n = 88$)	33 (37)	22 (25)	12 (14)	0 (0)
Other lower bowel diseases ($n = 30$)	10 (33)	6 (20)	5 (17)	0 (0)
Upper digestive tract diseases ($n = 58$)	23 (40)	16 (28)	7 (12)	0 (0)
Healthy individuals ($n = 41$)	10 (24)	5 (12)	4 (10)	1 (2)

[a] Number of positive feces. Numbers in parentheses stand for percentage (%).

[b] Formerly *Desulfomonas pigra*. Its prevalence is significantly higher in inflammatory bowel diseases as compared to healthy individuals or non-inflammatory bowel diseases (χ^2 test, $P < 0.05$).

searches, the prevalence of SRB in human clinical samples is certainly underestimated. The Test-kit Labège®, developed for the detection of SRB from environmental samples, proved to be a suitable medium for the detection of SRB from feces as well as from body fluids (Loubinoux, unpublished result). It has been shown by the manufacturer to grow environmental strains of SRB such as *D. desulfuricans* DSM 1926, *Desulfotomaculum nigrificans* DSM 574^T, *Desulfobacter postgatei* DSM 2034^T and *Desulfobulbus propionicus* DSM 2032^T. In our hands, it proved to be more sensitive than the commonly used Postgate's medium as six additional isolates of *Desulfovibrio* were detected in patients. Despite the abundant accompanying flora, the Test-kit Labège® permitted fast growth of the SRB from stool specimens as the mean time of detection was 2 days (versus 6 days in Postgate's medium). This may be related to the quality of the medium, but also to the mode of inoculation of samples that ensures the maintenance of strict anaerobiosis. As compared to Postgate's medium, the addition of acetate to lactate widens the detection spectrum to include slowly growing acetate metabolizing SRB such as *Desulfobacter* spp. Moreover, the presence of titanium citrate, which is a very efficient reducing agent (the redox potential of Test-kit Labège® is about -600 mV), allows a more rapid detection of most strains of SRB.

It is possible that some strains of SRB were not detected by the Test-kits Labège®, being overgrown by the accompanying flora or because of a low number in samples. However, the Test-kit Labège® is adapted to the growth of most of SRB, and all the positive flasks were identified by PCR as *D. piger*, *D. fairfieldensis* or *D. desulfuricans*. Despite the incubation time of 2 months, no additional species was evidenced. Thus, it is possible that our findings do correspond to the real human flora consisting almost exclusively of *D. piger* and *D. fairfieldensis*. *D. desulfuricans* was isolated once and has also been described in humans in a previous study [14], but it is likely uncommon in the intestinal tract. In most cases, *D. piger* and *D. fairfieldensis* were mutually exclusive. An association of both species was observed only once in a case of colonic cancer. To confirm the almost non-overlapping occurrence of *D. piger* and *D. fairfieldensis*, five colonies of SRB grown in solid Postgate's medium have been identified by PCR for each positive Test-kit Labège®. In each case, the same result was obtained and the five colonies belonged to the same species (data not shown). We have also made a follow-up of 10 patients (five SRB-positive and five SRB-negative) for 2 months and stools were cultured every week. For each patient, the same result (SRB-positive or SRB-negative) was obtained with all samples (data not shown). However, it would be interesting to follow the patients with IBD over a longer period of time to determine if the activity of the disease has a consequence on the population of SRB.

D. desulfuricans is commonly isolated from the environment, and has also been considered as the most prevalent species of *Desulfovibrio* in humans [9,11] until the recent description of *D. fairfieldensis* [16]. Thus, one may be surprised about the very low occurrence of *D. desulfuricans* in our population. Up to now, *D. piger* and *D. fairfieldensis* have been isolated solely from human samples. Thus, our results show that both species may be specific for the human intestinal tract. However, this remains to be determined by the specific search for these bacteria in other ecological niches. *D. piger* has been described only once [10], and was considered as an uncommon finding in humans. Our results indicate that it may be the most common SRB in the intestinal tract. However, this species has never been described in infectious processes. On the contrary, *D. fairfieldensis*, apparently less common in human feces, has been isolated outside the colonic lumen from blood and septic collections [13–16]. Thus, *D. fairfieldensis* may possess additional invasive properties as compared to other species of *Desulfovibrio*, which would explain its recovery from clinical samples. Interestingly, in our series of patients, the prevalence of *D. piger* in the feces is significantly higher in patients hospitalized for IBD (mostly CD) than in healthy individuals or in patients hospitalized for other pathologies. This may have two explanations: either *D. piger* has physiological characteristics that cause the onset of lesions and/or participate in the perpetuation of chronic inflammatory processes, or colonization by this species is favored by local conditions in IBD patients. The association of SRB with IBD has already been described [9]. *D. piger* has not been considered further since its first description in 1976 [10]. Thus, the finding that this bacterium, considered as a 'non-pathogenic' species, is a common inhabitant of the human intestinal tract and the most prevalent species of SRB in IBD patients is surprising. Additional studies should be conducted to elucidate the way in which *D. piger* may be implicated in these chronic inflammatory processes.

Acknowledgements

This work was supported in part by the Compagnie Française de Géothermie (Orléans, France) and by a FCT Grant POCTI 36562/ESP/2000 to ICP. We are indebted to the late Dr. Wee Tee (University of Melbourne, Australia) for kindly providing four strains of *D. fairfieldensis* (including strain ATCC 700045) and also to Prof. D. Raoult (University of Marseille, France) for providing one strain of *D. fairfieldensis*. We thank D. Meng and A.M. Carpentier (Laboratoire de Bactériologie-Virologie UMR CNRS 7565) for their excellent technical assistance, Dr. A. Dao and Prof. B. Fortier for providing feces from healthy individuals as well as the nurses of the Hepato-Gastro-Enterology Unit for their kindness.

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