High Prevalence of Methanobrevibacter smithii and Methanosphaera stadtmanae Detected in the Human Gut Using an Improved DNA Detection Protocol

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Abstract

Background: The low and variable prevalence of Methanobrevibacter smithii and Methanosphaera stadtmanae DNA in human stool contrasts with the paramount role of these methanogenic Archaea in digestion processes. We hypothesized that this contrast is a consequence of the inefficiencies of current protocols for archaeon DNA extraction. We developed a new protocol for the extraction and PCR-based detection of M. smithii and M. stadtmanae DNA in human stool.

Methodology/Principal Findings: Stool specimens collected from 700 individuals were filtered, mechanically lysed twice, and incubated overnight with proteinase K prior to DNA extraction using a commercial DNA extraction kit. Total DNA was used as a template for quantitative real-time PCR targeting M. smithii and M. stadtmanae 16S rRNA and rpoB genes. Amplification of 16S rRNA and rpoB yielded positive detection of M. smithii in 95.7% and M. stadtmanae in 29.4% of specimens. Sequencing of 16S rRNA gene PCR products from 30 randomly selected specimens (15 for M. smithii and 15 for M. stadtmanae) yielded a sequence similarity of 99–100% using the reference M. smithii ATCC 35061 and M. stadtmanae DSM 3091 sequences.

Conclusions/Significance: In contrast to previous reports, these data indicate a high prevalence of the methanogens M. smithii and M. stadtmanae in the human gut, with the former being an almost ubiquitous inhabitant of the intestinal microbiome.

Introduction

Archaea are environmental organisms that are associated with the mucosa in mammals [1–6]. In humans, Archaea are associated with the vaginal, oral, and intestinal mucosa [1–6]. Because of the fastidious nature of these strict anaerobes, most current knowledge about the archaeal flora of mammals is derived from DNA-based analyses. In the human gut, methanogenic Archaea metabolize major fermentation products, such as alcohols, short chain organic acids, carbon dioxide (CO2), and hydrogen (H2) [7]. Until recently, the diversity of gut methanogens was thought to be limited to two species: Methanobrevibacter smithii, the most abundant methanogenic Archaea found in the human gut [1–6,8–10], and Methanosphaera stadtmanae, which is seldom detected in the human gut. Both species can be detected by culture and PCR-based assays targeting the 16S rRNA and mcrA genes [8]. Recently, the DNA of additional methanogenic Archaea has been detected in human stool specimens, including DNA sequences that indicate the presence of a new order of methanogenic Archaea [2,11].

The predominance of M. smithii compared to M. stadtmanae can be explained by the fact that the genome of the former is more tailored to the gut environment with regards to metabolic versatility, genomic evolution ability, and persistence [9]. In the gut, M. smithii converts H2, CO2, and formate into CH4 using carbon as the terminal electron acceptor; this redox reaction sustains anaerobic respiration, which allows for the production of ATP [12,13]. This archaeon can also remove fermentation end products, such as methanol and ethanol, produced by other bacteria lacking a methanogenic pathway, while M. stadtmanae energy metabolism is limited to using hydrogen to reduce methanol to methane and is dependent on acetate as a carbon source [8]. Methanogenesis is of paramount importance in preventing the accumulation of gases and other reaction end products [9]. Accordingly, a metagenomic analysis of the gut flora in three healthy individuals found that M. smithii comprised up to 11.5% of the gut microorganisms [14]. However, 16S rRNA- and mcrA gene-based studies detected M. smithii with variable prevalence in less than half of the tested individuals and no M. stadtmanae; such results cannot reflect the actual quantity of these two Archaea in the human gut because of their specific association with the gut mucosa [14]. We hypothesized that the variable prevalence of M. smithii DNA in human stool specimens and the

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Competing Interests: The technical protocols herein presented have been patented by MH, DR and MD as inventors. Other authors declare no conflict of interest.

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failure to detect *M. stadtmanae* DNA in various studies may be due to limitations in the experimental protocols and to the relatively small samples comprising three or six individuals [14,15]. We aimed to establish an optimized protocol for the extraction and detection of archael DNA by exploiting the *M. smithii* and *M. stadtmanae* genome sequences [9,10].

**Materials and Methods**

2.1. Source of fecal samples

A series of 700 fecal specimens were prospectively collected to investigate the prevalence of *M. smithii* and *M. stadtmanae*. Stool specimens were stored at −20°C until used. A random subset of 50 specimens was further used to compare the DNA extraction method developed herein with a reference method. Only one stool specimen was collected per person, and for each person, sex, age, and presence of an enteric pathogen in the stool specimen responsible for diarrhea were documented. This study was approved by the local Ethics Committee.

2.2. Fecal DNA isolation

Approximately 1 gram of specimen was suspended in 10 ml of saline buffer and filtered through a Fecal Specimen Filtration Vial (Orion Diagnostica–Fumouze-Division Diagnostics, Levallois Perret, France). Aliquot of the filtrate (250 μl) was transferred into a sterile screw-cap Eppendorf tube containing 0.3 g of acid-washed glass beads (sterile screw-cap Eppendorf tube containing 0.3 g of acid-washed glass beads (≤106 μm, Sigma, Saint-Quentin Fallavier, France) and shaken to achieve mechanical lysis in a FastPrep BIO 101 apparatus (Qbiogene, Strasbourg, France) at level 6.5 (full speed) for 90 s. The supernatant was collected and incubated overnight at 56°C with 180 μl of T1 Buffer from the NucleoSpin Tissue Mini Kit (Macherey Nagel, Hoerdt, France) and 25 μl of proteinase K (20 mg/ml). After a second cycle of mechanical lysis as described above, the filtrate was incubated for 15 min at 100°C. Total DNA was then extracted using the NucleoSpin Tissue Mini Kit, according to the manufacturer’s procedure. Extracted DNA was eluted with 100 μl of elution buffer and stored at −20°C until used. A mock extraction performed using 250 μl of sterile water was used as a negative control for each batch of DNA extractions.

2.3. Real-time quantitative PCR assays

The targeted genes, probes, primer sequences (Eurogentec, Seraing, Belgium), and PCR product sizes for the four real-time PCR assays developed in this study are summarized in Table 1. The sequences of the four probes (Applied Biosystems, Courtaboeuf, France) and four primer pairs were designed using the *M. smithii* ATCC 35061 complete genome (GenBank accession number CP000678) and the *M. stadtmanae* DSM 3091 complete genome sequence (GenBank accession number NC 007681) (both exhibiting only one copy of the 16S rRNA gene and one copy of the rpoB gene) via the online Primer 3 program (http://www6.ncbi.nlm.nih.gov/BLAST). The specificities of the PCR primers and probes for the *M. smithii* ATCC 35061 and *M. stadtmanae* DSM 3091 16S rRNA and rpoB genes were verified in silico using the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The specificity was further experimentally ensured by incorporating the DNA extracted from 43 bacterial species representative of common gut inhabitants and enteric pathogens, including *M. stadtmanae* and *M. smithii* as positive controls (Table S2), in the real-time PCR protocol reported below. The primers and Taqman probes used for total bacterial real-time qPCR were adapted from the method previously described by Palmer and collaborators [16]. Real-time PCR assays were performed with a MX3000Q system (Stratagene, Amsterdam, The Netherlands) using the QuantiTect Probe PCR Kit (Qiagen, Courtaboeuf, France) with 5 pmol of each primer, probe labeled with FAM or VIC, and 5 μl of DNA (about 2 μg of total DNA) in a final volume of 25 μl. Positive controls (DNA extracted from *M. smithii* DSM6861 and *M. stadtmanae* DSM 3091 strains, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), an extraction control, no-template controls, and a quantification scale from 10^−7 to 10 target copies were utilized in each reaction plate. The 16S rRNA and rpoB gene copies were quantified in a duplex assay, and total bacteria were used in

| Table 1. Real-time PCR primers and probes sequences for detecting *M. smithii* and *M. stadtmanae* 16S rRNA and rpoB genes and for detecting all bacteria (Totbact). |
|----------------------------------|------------------|------------------|
| **Organism**                     | **Assay**        | **Primer/probe name and sequence (5’-3’)** | **Dye** | **Product size (bp)** |
| *M. smithii*                     | 16S rRNA         | Smit.16S-740F, 5’-CCGGGTATACTTACCGGATTC-3’ | FAM (MGB) | 123 |
|                                  |                  | Smit.16S-862R, 5’-CTCCAGGGATAGTTGGA-3’       |         | 70 |
|                                  |                  | Smit.16S FAM, 5’-CCGTCAAAATCGTTCAGTACG-3’    |         | 97 |
|                                  | rpoB             | MsrpoBF, 5’-AAGGATTTGACCCAAACAC-3’           |         | 95 |
|                                  |                  | MsrpoBR, 5’-GACCACAGTTGACCCCTCTGG-3’         |         | 95 |
| *M. stadtmanae*                  | 16S rRNA         | Stadt.16SF, 5’-AGGAGCGACAGCAAGAATGAT-3’      | FAM (MGB) | 123 |
|                                  |                  | Stadt.16SR, 5’-CCGGGTATCTAATCCGGTTC-3’       |         | 70 |
|                                  | rpoB             | stadtrpoBF, 5’-TCTTTGGATTTTTGCTGCGA-3’       | FAM (MGB) | 123 |
| All bacteria                     | **Totbact**      | Bact8FM, 5’-AGAGTTGTACMTGTTGCTACG-3’         |         | 327 |
|                                  |                  | Bact515R, 5’-TTACCGCGCGXCGGCGACACG-3’        | FAM (MGB) | 123 |
|                                  |                  | Bact338K, 5’-CCAKACTCTACGGAGGAGGACAC-3’      | FAM (MGB) | 123 |

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simplex real-time qPCR as a bacterial DNA extraction control. The PCR amplification program was 95°C for 15 min, followed by 45 cycles of 95°C for 30 s and 60°C for 1 min. All DNA samples were tested in duplicate. Results are expressed as the number of 16S rRNA and rpoB copies per gram of feces. Plasmid quantification was carried out by inserting a chimeric nucleotide fragment into the pCR II plasmid (TA cloning Kit Dual Promoter [pCRII] Invitrogen, Carlsbad, CA, USA), as previously described [17]. The chimeric fragment contained real-time PCR targets for M. smithii and M. stadtmannae 16S rRNA and rpoB genes, together with a universal 16S rRNA bacterial target gene adapted from the Streptomyces sudanensis strain SD504 sequence (GenBank accession number EF515876) as an internal control to monitor the absence of PCR inhibition.

2.4. Sequencing

To sequence the M. smithii and M. stadtmannae DNA in human feces, real time-PCR products of the 16S rRNA gene were sequenced for 30 randomly chosen specimens using the primers Smit.16S-740F, Smit.16S-862R, Stadt_16SF and Stadt_16SR (Table 1). PCR products were purified and sequenced using the BigDye Terminator 1.1 Cycle Sequencing kit and the 3130 genetic analyzer (Applied Biosystems). Negative controls were employed for each assay. Sequences were analyzed using the Seqscape program (Applied Biosystems), and similarity values were determined using the online BLAST program at NCBI.

2.5. Protocol sensitivity

To monitor the sensitivity of our extraction protocol, three stool specimens presenting low loads of M. smithii 16S rRNA and rpoB genes supplemented with 100 μl of M. smithii DSM 861 suspension were used. For all specimens in both the 16S rRNA and rpoB assays, the sensitivity was calculated by subtracting the gene copy number present before addition of M. smithii to that obtained after and then dividing by the gene copy number obtained for 100 μl of a pure suspension of M. smithii. The protocol sensitivity presented is the average of the three percentages obtained.

2.6. Comparison with the reference DNA extraction protocol

Total DNA was isolated from 50 stool specimens using the extraction protocol described above in parallel with the QIAamp Stool DNA mini kit protocol (Qiagen), as previously reported [14]. The latter kit was used as a gold standard, as a literature review indicated that this kit was used in almost all recent studies (2005–2008) dealing with DNA extraction from animal and human stools. We then compared the numbers of M. smithii 16S rRNA and rpoB gene copies per gram of stool detected by each DNA extraction protocol. Numerical data were analyzed using EPIINFO version 3.4.1 software (Centers for Disease Control and Prevention, Atlanta, GA). P values were used to assess statistical significance when comparing the two methods and were calculated using the non-parametric Kruskal-Wallis test for two groups. A P value<0.05 was considered to be significant.

Results

3.1. Stool specimens

Stool specimens were collected from 700 persons [408 males (aged 1 day to 95 years) and 292 females (aged 1 month to 98 years)] (Table S1). The stool specimens of 643 of these patients had been submitted for the identification of enteric pathogens; the remaining 57 stool specimens had been submitted for the determination of Staphylococcus aureus and Salmonella spp. intestinal carriage in healthy adults without diarrhea and no history of enteric disease in the past six months. No enteric pathogens were identified in the healthy adults, whereas enteric pathogens were cultured from 4 of the remaining 643 patients, including Salmonella enterica in 2 patients, Staphylococcus aureus in 1 child, and Shigella sonnei in 1 patient (Table S1).

3.2. Real-time PCR

In-silico analysis using the online BLAST program at NCBI indicated that the M. smithii and M. stadtmannae real-time PCR systems were specific for the two species. These data were experimentally confirmed by the fact that the systems did not detect the DNA extracted from 43 other enteric organisms in the presence of positive controls, with the exception of the homologous methanogenic species. Negative results were obtained for all negative controls in the assay. Our protocol demonstrated an extraction efficiency of 89.82% for the 16S rRNA assay and 94.04% for the rpoB assay. The present study, 670/700 stool specimens were positive for the M. smithii 16S rRNA or rpoB genes (95.7%), including 133 specimens that were positive for the 16S rRNA gene only. The 16S rRNA and the rpoB gene copy numbers varied from 10.9 ± 1.45 × 10¹¹ and from 2.22 ± 2.40 × 10¹⁰ copies per gram of stool, respectively (Table 2). The internal positive control was detected in 29/29 (100%) stool specimens that remained negative for M. smithii DNA.

Among these 700 stool specimens, 206 (29.4%) were positive for M. stadtmannae DNA, including 71 that were positive for the 16S rRNA gene only. In all cases, M. stadtmannae DNA was detected in association with M. smithii DNA (Table S1). The 16S rRNA and the rpoB gene copy numbers varied from 12 ± 1.45 × 10¹⁰ and from 37.9 ± 2.74 × 10⁰ copies per gram of stool, respectively (Table 2). The internal control was detected in 44/44 (100%) randomly

| Table 2. Mean, standard deviation, median, and maximal and minimal values of the copy numbers of M. smithii and M. stadtmannae 16S rRNA and rpoB genes per gram of stool in the positive specimens for each assay. |

|                     | M. smithii | M. stadtmannae |
|---------------------|------------|----------------|
|                     | 16S rRNA assay | rpoB assay     | 16S rRNA assay | rpoB assay     |
| Minimum             | 1,09E+01    | 2,22E+00       | 1,20E+01       | 3,79E+01       |
| Maximum             | 1,45E+11    | 2,40E+10       | 1,45E+10       | 2,74E+09       |
| Target copy number Mean | 2,33E+09    | 6,46E+08       | 1,80E+08       | 6,56E+07       |
| Standard deviation  | 1,26E+10    | 2,70E+09       | 1,25E+09       | 3,40E+08       |
| Median              | 4,20E+04    | 1,72E+04       | 9,30E+03       | 2,66E+04       |

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selected stool specimens that lacked M. stadtmanae DNA PCR product.

No statistical correlation was observed between M. smithii/M. stadtmanae detection or load and the presence of an enteric pathogen. No statistical difference was detected in M. smithii/M. stadtmanae detection and load between the 57 healthy individuals and the individuals who submitted stool samples for pathogen identification.

3.3. Sequencing

All negative controls lacked PCR products. Direct sequencing of the 30 16S rRNA gene PCR products yielded a BLAST similarity of 99% with the reference M. smithii ATCC 35061 16S rRNA gene sequence (GenBank accession NC_009515) and a BLAST similarity of 100% with the reference M. stadtmanae DSM 3091 16S rRNA gene sequence (GenBank accession CP000102).

Interestingly, M. smithii DNA was detected in 2 specimens collected from children aged 1 and 2 years, with estimated numbers of 10 and 776 M. smithii per gram of stool, respectively. A sequence similarity of 99% compared to the M. smithii reference strain was obtained for both children.

3.4. Comparison of DNA extraction protocols

DNA extracted using the QIAamp Stool DNA mini kit was positive for M. smithii 16S rRNA and rpoB genes in 44/50 (90%) and 33/50 (66%) specimens, respectively, by quantitative real-time PCR. DNA extracted using the protocol described herein was positive for M. smithii 16S rRNA and rpoB genes in 50/50 (100%) and 49/50 (98%) specimens, respectively. Per 1 g of stool, the extraction protocol developed herein yielded 100- to 1,000-fold more gene copies than the reference extraction protocol (P≤10^-5) (Table 3).

Discussion

Based on a metagenomic analysis of stool samples collected from three healthy adults, M. smithii DNA was estimated to comprise up to 11.5% of the total human gut microbiome [14]. Such a high inoculum is in agreement with the paramount role of this archaeon in the detoxification of digestion by-products [9], but contrasts with the relatively low (<5%) rate of detection observed in studies based on either isolation or PCR-based detection of specific DNA sequences [5,14,16].

Here, we employed an optimized protocol for the extraction and specific PCR-based detection of M. smithii DNA in stool samples and demonstrated that M. smithii DNA can be detected in the stool of almost all individuals (95.5%) and M. stadtmanae in 29.4% of individuals. The fact that negative results were obtained for the negative controls and the fact that all experiments were conducted under strict rules to prevent laboratory contamination, together with the recovery of original sequences, indicate that the positive detection of M. smithii and M. stadtmanae DNA achieved in this study was not due to specimen contamination. Moreover, the specificity of the real-time PCR systems developed herein was ensured by in-silico analysis and experimental testing using DNA extracted from common human gut inhabitants and pathogens. A high, 99-100% sequence similarity of real-time PCR products with that of reference sequences finally ensured the specificity of the real-time PCR systems herein reported. Likewise, detection of the internal positive control in all specimens negative for M. smithii or M. stadtmanae eliminated the possibility of PCR inhibition. Therefore, the data likely accurately estimate the prevalence of both M. smithii and M. stadtmanae in the population studied. Comparison of the DNA extraction protocol developed herein with the protocol previously used for metagenomic analysis of the human gut [14,16] revealed a two to three log difference in the quantity of archaeon DNA, with greater detection achieved with the new protocol. This difference in performance was likely due to an optimized combination of previously reported lysis methods [14,15,18,19]. The fact that members of the family Methanobacteriales, such as M. smithii and M. stadtmanae, have a proteinase K-resistant cell wall [20], suggests that double mechanical cell lyses using glass beads was decisive in the efficiency of DNA extraction. This fact was further illustrated by the detection of low DNA loads (Tables 2 and 3), whereas the sensitivity was measured to be ≥10^7 cells per gram of feces in a previous study [11]. Such a huge difference in the sensitivity of M. smithii DNA detection may have influenced previously published data on the prevalence of M. smithii in stool.

This protocol was also shown to be effective for DNA extraction from Methanobacterium tuberculosis present in stool [21].

In most previous studies, the universal 16S rRNA gene was used as a suitable target for the molecular detection of archaeal DNA present in the human and animal gut [14,16,18,22]. The mcrA gene, which encodes the methyl-coenzyme M reductase in M. smithii, has also been used [15]. However, M. stadtmanae mcrA gene-derived primers failed to detect M. stadtmanae in a total of 207 individuals [11]. We therefore used the rpoB gene, which encodes the β subunit of RNA polymerase and is one of the core genes shared by Bacteria and Archaea [23,24], the rpoB gene has been previously used to infer phylogenetic relationships between Archaea and was recently demonstrated to correlate with DNA:DNA hybridization data, as well as the average nucleotide identity among prokaryotes [23,25,26]. In the present study, the specificity of M. smithii and M. stadtmanae detection was confirmed by evaluating two independent, universal molecular targets (16S rRNA and rpoB genes) using specific and highly sensitive quantitative real-time PCR. The latter gene exhibits a 12% sequence divergence between M. smithii, conferring a higher specificity than the 16S rRNA gene system. Moreover, sequencing of the PCR products obtained for M. smithii

Table 3. Copy numbers of M. smithii 16S rRNA and rpoB genes per gram of stool obtained using the protocol reported herein versus those obtained via the reference Qiagen protocol for M. smithii DNA extraction from human stool (1).

| Assay | Mean | Standard Deviation | Median | Maximum | Minimum | Mean | Standard Deviation | Median | Maximum | Minimum | P value(1) |
|-------|------|-------------------|--------|---------|---------|------|-------------------|--------|---------|---------|------------|
| 16S rRNA | 4.11e+09 | 1.23e+10 | 7.54e+05 | 7.43e+10 | 100 | 3.02e+07 | 1.25e+08 | 1.13e+03 | 7.20e+08 | 0 | ≤0.00001 |
| rpoB | 1.48e+09 | 3.80e+09 | 2.94e+06 | 1.65e+10 | 0 | 1.01e+07 | 4.11e+07 | 2.20e+03 | 2.27e+08 | 0 | ≤0.00001 |

1) P values were determined using the non-parametric Kruskal-Wallis test for two groups.

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confirmed the detection specificity of the real-time PCR assays, as sequence similarity values were 99% compared to the reference *M. smithii* sequence. Likewise, sequence similarity values were 100% for *M. stadtmanae*.

Data herein presented shed new light on the biology of methanogenesis in the human gut. While breath methane measurement has a high positive predictive value, its sensitivity was lower than that of real-time molecular detection of methanogens in healthy individuals, the breath methane test being positive only for *M. smithii* inoculum >10^7 organisms per gram of stool [27]. Indeed, breath methane measurement typically yields a prevalence of 40% [27,28], in contrast to the 95.7% of methanogen DNA in our study. Despite the fact that molecular test, contrary to the breath methane test, detects both alive and dead methanogens, data herein reported indicate that gut methanogens are detectable in almost all individuals, regardless of them being healthy individuals or suffering from intestinal disease. Moreover, the breath methane test cannot determine the precise microbial source of methane in the gut; the fact that we detected *M. stadtmanae* in addition to *M. smithii* in about one-third of individuals, and that other methanogen DNAs have been detected in some individuals [15,27], suggests that molecular determination of the gut methanogen flora is essential for any study dealing with the biology and the dynamics of methanogenesis in both healthy individuals and in patients presenting with intestinal disease under widespread antibiotic and dietary changes. We did not detect *M. smithii* and *M. stadtmanae* in less than 5% of individuals; whether this lack of detection reflects the limit of molecular detection or true absence of both methanogens, remains to be determined. Performing a breath methane test may help resolve this issue and give a basis to continue molecular detection of methanogens in such individuals. Our study indicates for the first time that methane production could be due to at least two archael species in the same individual but the *M. smithii* and *M. stadtmanae* DNA loads were highly variable among specimens. We did not collect several specimens per individual in a time-dependent manner, and therefore we cannot ascertain whether the *M. smithii* and *M. stadtmanae* DNA loads remain constant over time for an individual or vary under physiological and pathological circumstances. Interestingly, *M. smithii* DNA was detected in all stool specimens collected from 16 children younger than 2 years of age, including one specimen collected from a 2-week-old infant that was negative using the reference extraction protocol. Therefore, our results complete previous data demonstrating the transient detection of *M. smithii* DNA during the first 5–120 days of life in stool samples obtained from 7/14 children younger than one year of age [16]. These data indicate that the acquisition of *M. smithii* is an early event in newborns, resulting from maternal or environmental exposure. *M. smithii* has been detected in the vaginal flora, including that of 3/13 pregnant women [2,16].

The DNA extraction protocol and highly specific and sensitive method of detection and quantification developed in the present study offer new tools on which to base the optimized, broad-spectrum molecular detection of *Archaea* embedded in the complex microbiomes associated with the mammalian mucosa. This protocol may aid in identifying additional species of *Archaea* and in monitoring modifications in the archaela flora under various physiological, pathological, and therapeutic circumstances.

**Supporting Information**

**Table S1** The Ct values for the real-time PCR detection of *M. smithii* and *M. stadtmanae* in human stools. Found at: doi:10.1371/journal.pone.0007063.s001 (0.10 MB XLS)

**Table S2** List of bacteria tested for the specificity of real-time PCR. Found at: doi:10.1371/journal.pone.0007063.s002 (0.05 MB XLS)

**Author Contributions**

Conceived and designed the experiments: MH DR MD. Performed the experiments: BD AEK. Analyzed the data: BD MH DR MD. Contributed reagents/materials/analysis tools: DR MD. Wrote the paper: BD MH DR MD.

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