Abundance of sulphate-reducing bacteria in human feces is overestimated by QPCR that targets the Desulfovibrio 16S rRNA gene

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Running title: Quantification of SRB in human feces

Keywords: gut, adenosine-5’-phosphosulfate reductase (aps), dissimilatory (bi)sulfite (dsrA), hydrogen sulphide and SRB.

Word count:
Abstract (limit 50 words): 49
Total (if possible < 1000 words): 1344
Abstract

The dominant group of sulphate-reducing bacteria (SRB) in humans is *Desulfovibrio* and Quantitative PCR (QPCR) targeting the 16S rRNA gene is often used. We show the assay overestimates SRB abundance in feces from 24 adults compared to QPCR assays using primers targeting two genes involved in SRB energy metabolism.
There is growing interest in the quantification of sulphate-reducing bacteria (SRB) in humans because of reports suggesting SRB to be involved in the aetiology of gastrointestinal diseases (10, 18). The principal by-product of SRB metabolism is hydrogen sulphide (H$_2$S), which can be toxic to epithelial cells in the colon where it is mainly produced. At higher levels H$_2$S can inhibit butyrate oxidation (20), phagocytosis and bacterial killing (11), and induces hyperproliferation and metabolic abnormalities in epithelial cells (5). H$_2$S is also produced endogenously by colonocytes (21) and is physiologically active in the brain, heart, vasculature, urogenital system and gastrointestinal tract at non-toxic levels (8, 25, 26). Ulcerative colitis patients were reported to have higher levels of H$_2$S (19) and a higher abundance of SRB (12) in their feces. Using the *Desulfovibrio* 16S rRNA gene for detection of SRB in human feces has revealed a higher abundance in elderly people compared to healthy adults (9), a positive correlation between *Desulfovibrio* abundance and smoking (14), and no difference (2) or a decrease in *Desulfovibrio* in colorectal cancer patients (22) compared to healthy individuals. Therefore, to determine the role of SRB in gastrointestinal health an accurate estimate of the abundance of SRB is vital.

For quantification of SRB in environmental samples three genes, 16S rRNA, adenosine-5’-phosphosulfate reductase (*aps*) and dissimilatory (bi)sulfite (*dsr*A), are generally targeted (3, 24). The two genes, *aps* and *dsr*A, are involved in the energy metabolism of SRB and have been identified as reliable gene markers for SRB (24). The 16S rRNA gene is however considered inadequate in environmental samples because SRB are found in different phyla in the phylogenetic tree (4, 24). Therefore, the 16S rRNA gene cannot cover all the different phyla and underestimates the SRB abundance in environmental samples (3, 24). This may however not be the case for
human fecal samples as SRB of the genus *Desulfovibrio* can occur in large numbers in the gut and has been identified as the main genus for SRB in humans (9, 12).

In this study we examine, using quantitative real time PCR (QPCR), the accuracy of using the 16S rRNA gene to quantify SRB in human feces by comparing the widely used 16S rRNA primer pair that targets the *Desulfovibrio* (9) with two QPCR assays targeting the SRB functional genes, *aps* and *dsrA*, found to give reliable quantifications of SRB in environmental samples.

Human fecal samples were collected from 24 healthy individuals, including 14 males and 10 females with an average age of 53.7 years (range 33-67), who had not had any antibiotic treatment during the past 6 months. DNA was extracted from these samples using the repeated beat-beading and column clean-up method described by Yu and Morrison (27). *Desulfovibrio*, *aps* and *dsrA*, and total number of bacteria in human feces were quantified using QPCR. Quantifications were performed using 1× Ssofast Evagreen Supermix fluorescent nucleic acid dye (Bio-Rad Laboratories, Hercules, California, USA), 0.4µl bovine serum albumin (BSA) (Promega, Madison, Wisconsin, USA). Primers (concentration): Total bacteria (175nM), 1114f (5`-CGGCAACGAGCGCAACCC-3`) and 1275r (5`-CCATTGTAGCACGTGTGTAGCC-3`) (6); *aps* (400nM) *aps*3f (5`-TGGCAGATCATGWTYAAYGG-3`) and *aps*2r (5`-GGGCCGTAACCRTCYTTRAA-3`) (modified from 7); *dsrA* (400nM) Dsr1F (5`-ACSCACTGGAACGAGCGGCGG-3`) and Dsr1R (5`-GTGGMRCCCTGCAKRTTGG-3`) (16); *Desulfovibrio* (16S rRNA) (300nM) DSV691F (5`-CCGTAGATATCTGGAGGAACATCAG-3`) and DSV826R (5`-ACATCTAGCATCCATCGTTTACAGC-3`) (9). For quantification a total of 10-30 ng of template DNA was used and cycling was performed in a Chromo-4
thermocycler (MJ Research Inc., Waltham, Massachusetts, USA). Reactions for *aps* assays contained 1µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri, USA) instead of BSA. The PCR cycling conditions were as follows: 98 °C for 4 min followed by 35 cycles of 98 °C for 30 sec, 58-65°C for 15-30 sec (total bacteria: 60 °C for 20 sec; *aps*: 58 °C for 30 sec; *dsrA*: 65 °C for 15 sec; *Desulfovibrio* 62 °C for 30 sec) and 72 °C for 30 sec. Elongation was followed by fluorescence acquisition, however a further elongation step at 83°C for 15 sec was performed before fluorescence acquisition for *dsrA*, and a final meltcurve analysis was performed after completion of all cycles with fluorescence acquired at 0.5 °C intervals between 55 and 95°C to verify that only the expected fragment was amplified. PCR product was also visualised on a 1.5% agarose gel. Non-template controls were included and assays were performed in technical triplicate by analyzing the same DNA sample in 3 independent reactions. An 8-series of 10-fold dilutions of a sample derived plasmid construct (Topo chemical competent cells, Invitrogen) containing the target amplicon were analyzed in parallel with DNA samples for estimation of absolute abundance and PCR efficiency for all assays. Results were analysed with the Opticon Monitor software (Version 3.1; Bio-Rad Laboratories) for total abundance estimates. All calculations were done using an assay specific PCR efficiency. A specificity test was performed on the 16S rRNA gene primers using a clone library. Twenty four clones were sequenced using a 96-capillary 3730xl DNA Analyzer and putatively identified using Basic Local Alignment Search Tool (1). Statistical analysis was performed using the Primer 6 with Permanova+ package (PRIMER-E Ltd, Plymouth, UK). Natural logarithm normalized data was used for statistical analysis of absolute quantities.
The absolute quantification of SRB in human feces using the 3 primer sets showed an average abundance (copies x gram wet feces\(^{-1}\) (range)) of: *aps* 3.26x10\(^6\) (2.54x10\(^4\) – 1.19x10\(^7\)); *dsrA* 2.21x10\(^6\) (1.48x10\(^5\) – 1.01x10\(^7\)) and *Desulfovibrio* 4.53x10\(^7\) (1.36x10\(^6\) – 2.26x10\(^8\)). These abundances revealed a significantly higher absolute abundance (Figure 1) of *Desulfovibrio* compared to *aps* (P=0.00005) and *dsrA* (P=0.00001) but no significant difference was observed between *aps* and *dsrA*. Similar differences were found when data was analyzed relative to the total number of bacteria using Qbase\(^+\) (13, 23) (data not shown). The PCR efficiency for the assays was as follows: Total bacteria: 103%; *Desulfovibrio*: 103%; *dsrA*: 105% and *aps*: 99%. These efficiencies were similar for both plasmid and stool-derived DNA. The clone library revealed that 75% (18 clones) were 98 – 100% similar and 17% (4 clones) were between 92% - 97% similar to known *Desulfovibrio* sequences, whereas 8% (2 clones) were putatively identified as *Papillibacter cinnamivorans*. It was also noted that all 24 healthy volunteers had detectable numbers of SRB.

This study supports the evidence that *Desulfovibrio* is the dominant genus of SRB found in stool from humans based on the clone library and suggests that using the *Desulfovibrio* 16S rRNA gene overestimates the abundance of SRB in human feces compared to SRB abundances estimated using the *aps* or the *dsrA* gene. Hence caution has to be taken in analyzing and reporting SRB abundance when quantifications are performed using the 16S rRNA gene. However, the abundances of *aps* and *dsrA* also vary between individuals (Figure 1) because some SRB may carry both genes and some only one of them (3). According to other studies, both the *aps* and *dsrA* are reliable genes suitable for quantification of SRB populations and they are specific for the SRB energy metabolism. The absolute quantities of *Desulfovibrio*, using the 16S rRNA primers, in this study are similar to the findings of Fite *et al.* (9)
and with PCR efficiencies around 100% (99% - 105%) we are confident that the 16S rRNA gene primers used in this study overestimate the abundance of SRB in human feces. That is opposite to what is found in environmental samples but in-line with the general conception that often 16S rRNA primers do overestimate abundances due to a higher copy number of the 16S rRNA gene compared to aps and dsrA. According to the Ribosomal RNA Operon Copy Number Database (15, 17) the genus Desulfovibrio has on average 4.5 copies of the 16S rRNA gene. Another reason for the 16S rRNA gene primers overestimating the abundance of Desulfovibrio is the specificity of the primers. The clone library showed that the primers did amplify two fragments that did not match Desulfovibrio even though in silico tests showed the 16S rRNA gene primers to have several mismatches. In conclusion, the overestimation of abundance observed when using 16S rRNA gene primers compared to aps and dsrA primers in this study is almost certainly due to unspecific priming of the 16S rRNA gene primers and a higher copy number of the 16S rRNA gene compared to the aps and dsrA gene.
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Figure 1: Abundance of SRB (mean ± SD) in feces from 24 healthy volunteers measured with QPCR using primers that target the 16S rRNA gene of Desulfovibrio spp. (Δ), the adenosine-5'-'phosphosulfate reductase (aps) gene (○) and the dissimilatory (bi)sulfite (dsrA) gene (▼). QPCR estimates of total number of bacteria are also represented (●).