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Variability of Oxalobacter Formigenes and Oxalate in Stool Samples

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Abstract

Purpose—The intestinal organism *Oxalobacter formigenes* is unique in using oxalate as its primary carbon and energy source. Intestinal colonization with *O. formigenes* may have clinical significance by decreasing intestinal oxalate and its absorption, thereby influencing the concentration of oxalate in plasma and urine, and the development of calcium oxalate stone disease. Because the oxalate content of the diet varies considerably, we hypothesized that the number of *O. formigenes* and amount of oxalate would vary in feces.

Materials and Methods—To enumerate the number of *O. formigenes* in feces an accurate and reproducible real-time polymerase chain reaction assay was developed to quantify *O. formigenes* DNA. Stool samples were obtained from 10 colonized individuals to determine the levels of *O. formigenes* by this assay and the oxalate content by ion chromatography.

Results—Concentrations of *O. formigenes* ranged from lower than the limit of detection of 5×10^3 to 1.04×10^9 cells per gm stool. The total oxalate content of stool samples varied from 0.1 to 1.8 mg/gm and fecal water oxalate varied from 60 to 600 μM . All parameters measured varied within each stool collection, among stool collections on different days and among individuals. Notably in 7 of 10 individuals at least 1 stool sample contained no detectable *O. formigenes*. In addition, 7 of 10 subjects had a fecal colonization of less than 4×10^4 per gm stool.

Conclusions—This study demonstrates that there is intrastool and interstool sample variability in the amount of *O. formigenes* measured by real-time polymerase chain reaction that did not correlate with the quantity of oxalate in stool. Most subjects had a fecal colonization of less than 4×10^4 per gm stool.

Keywords

kidney; kidney calculi; *Oxalobacter formigenes*; calcium oxalate; intestines

Oxalobacter formigenes is a specialized anaerobic organism that can colonize the human large intestine. It is a specialist since it uses oxalate as its main carbon and energy source.^{1,2} It relies primarily on oxalate ingestion by the host for survival, although it is possible that small amounts of oxalate secreted from the body into the intestine help sustain colonization.³ It was hypothesized that intestinal colonization with this organism has clinical significance because it may limit intestinal oxalate absorption and under certain conditions it may promote the secretion of oxalate into the gut.^{4–6}

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Study received institution review board approval.

We estimated that the mean oxalate intake is approximately 150 mg per day but there is a wide range due to the variety and quantity of foods eaten, and variability in the oxalate content of foods.⁷ A possible repercussion is that intestinal contents and feces also vary in their levels of *O. formigenes* and oxalate. There is some evidence that the number of *O. formigenes* in feces can be influenced by dietary oxalate. Doane et al reported that fecal levels of *O. formigenes* in colonized individuals increased 5 to 14-fold when the diet was supplemented with a quantity of spinach containing 1,500 mg oxalate.⁸

The number of *O. formigenes* organisms in feces was previously assessed by measuring cleared zones in defined agar medium containing calcium oxalate crystals.¹ The reported range using this approach was 3×10^5 to 3×10^8 cells per gm feces.^{8,9} A quantitative PCR technique using a competitive DNA template for enumerating *O. formigenes* was previously described.¹⁰ In this study we developed a real-time PCR procedure for quantifying *O. formigenes*. We examined the variability of oxalate and number of *O. formigenes* within and between stool samples from 10 colonized individuals. This technique is more accurate and less time-consuming than the competitive PCR procedure.

MATERIALS AND METHODS

Subjects

Potential subjects who denied the current presence or a history of any disease that may affect intestinal function, hepatic metabolism or renal function were screened for participation in the study, which was approved by the institutional review board. For screening purposes each provided a stool sample to test for colonization with *O. formigenes* by conventional PCR.¹¹ Ten healthy, adult nonkidney stone formers colonized with *O. formigenes*, including 7 males and 3 females with a mean $\pm$ SD age of 29 ± 4 years, were recruited for study. None of the subjects had received antibiotics or probiotic preparations in the 6 months before enrollment.

Samples

Participants provided fecal samples within 4 hours of passage for 5 consecutive days. Three 200 mg aliquots were taken from 3 regions of each stool sample and processed immediately. They were initially centrifuged at $16,100 \times$ gravity for 20 minutes at room temperature to obtain a 5 to 10 μ l fecal water sample. DNA was extracted from the remaining sample using a Qiagen^{TM} DNA Stool Kit. After adding lysis buffer and heating the suspension for 5 minutes at 70C a 50 μ l fecal homogenate sample was taken for total oxalate analysis. DNA extracted was quantified using a Beckman^{TM} DU 640i spectrophotometer.

Oxalate Analysis

Fecal water and fecal homogenate samples were acidified below pH 1 with ultra pure hydrochloric acid (Fluka, Buchs, Switzerland) before storage at -70°C . Before oxalate analysis fecal samples were diluted 100 to 500-fold in distilled water, followed by centrifugation at $16,100 \times$ gravity for 20 minutes at room temperature. Oxalate in fecal samples was determined by ion chromatography (Dionex, Sunnyvale, California) with suppressed conductivity detection. Ion chromatography equipment consisted of a ED50 conductivity detector, GP50 gradient pump, AS50 autosampler, ASRS-ULTRA 2 mm suppressor (Dionex) and IonPac® AS4A-SC 2×250 mm ion exchange column with guard column. Oxalate was baseline separated from other anions using 0.7 mM carbonate/1.7 mM bicarbonate at a flow rate of 0.5 ml per minute.

Primers

Primers were developed using SeqWeb 2.1 (Accelrys®), which amplified a 164 bp sequence of the oxalyl coenzyme A decarboxylase (Oxc) gene. The specificity of these primers was confirmed by examining their homology with all other bacterial sequences deposited at the National Center for Biotechnology Information gene BLAST website (<http://www.ncbi.nlm.nih.gov/BLAST>). The forward primer consisted of 21 nucleotides with the sequence CGACAATGTAGAGTT-GACTGA (GC content 42.8% and melting temperature 59.5C). This included a 17 bp overlap with the forward primer used by Sidhu et al.¹⁰ The reverse primer, consisting of 19 nucleotides, was CGTGTTGTTCGTGACGAA (GC content 52.6% and melting temperature 60.5C).

PCR

Real-time PCR was performed using the DNA Engine Opticon® 2, version 2.02. All reactions were performed in 8 Low Tube Strips and closed with Flat Cap Strips (Bio-Rad™). PCR mixture (50 µl) contained 25 µl 2 × QuantiTect® SYBR® Green PCR Master Mix, forward and reverse primers, each at a final concentration of 0.25 µM, and 100 ng template DNA. Bovine serum albumin was added at 0.1 µg/µl to enhance PCR robustness. Uracil-N-glycosylase was added at 0.5 U per reaction to eliminate any deoxyuridine monophosphate containing products, which may result from carryover contamination. Optimization resulted in real-time cycling conditions of 50C for 2 minutes (carryover prevention) and 95C for 15 minutes (PCR initial activation step), followed by 45 cycles of denaturation at 94C for 15 seconds, annealing at 57C for 30 seconds and extension at 72C for 30 seconds. Each cycle was completed with melting peak analysis (55C to 95C) to confirm amplicon specificity and the lack of mispriming products, including primer dimers. An NTC and standards were included in each plate. If the NTC was positive, data were discarded and the reaction was repeated until the NTC was negative. A threshold line was drawn as low as possible above the noninformative fluorescent noise band, enabling the production of a standard curve with a correlation coefficient close to 1.¹²

For conventional PCR AccuPrime™ SuperMix I was used according to manufacturer instructions with the same primers as for real-time PCR. PCR cycling conditions consisted of initial denaturation at 94C for 2 minutes, followed by 35 cycles of denaturation at 94C for 30 seconds, annealing at 55C for 30 seconds and extension at 72C for 30 seconds. Amplification products were analyzed by agarose gel electrophoresis.

Standard Curves

O. formigenes, strain HC-1 (OxThera, Gainesville, Florida) was grown overnight at 37C in Medium B.¹ Cells were counted by flow cytometry using a B-7277 Bacterial Counting Kit (Molecular Probes™). Cells were also harvested by centrifugation and DNA extracted using a Qiagen DNA Extraction Kit, permitting the calculation of the DNA content per cell.

RESULTS

As determined by flow cytometry, the average number of cells present was $8.6 \pm 1.4 \times 10^8$ /ml. The linear dynamic range of the real-time PCR assay was determined by serial dilution of *O. formigenes* DNA extracted from overnight cultures. Figure 1 shows the relationship of the number of *O. formigenes* to C_t . The assay was found to be linear over a 5 log₁₀ range with a limit of detection of 5 cells or 5×10^3 cells per gm stool. Assay precision was tested by repetitive analysis of stool containing 2.2×10^4 *O. formigenes* per gm in 6 preparations. The intra-assay coefficient of variation was 1.3% for C_t and 14% for the number of bacteria enumerated. Method accuracy was tested by spiking stool that was negative for *O. formigenes* by culture and PCR tests (fig. 2). Mean recovery was 96% to 110% of the DNA added. Assay specificity

was confirmed using DNA (American Type Culture Collection, Manassas, Virginia) isolated from organisms reported to degrade oxalate, including *Acinetobacter* species, *Bacillus subtilis*, *Bifidobacterium breve*, *B. infantis*, *Enterococcus faecalis*, *Lactobacillus acidophilus* and *Providencia stuartii*, and those not known to degrade oxalate, including *Clostridium perfringens*, *Escherichia coli*, *Eubacterium limosum*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

The effect of storage at different temperatures on the stool content of *O. formigenes* DNA was examined. After 24 hours at room temperature *O. formigenes* DNA recovered from 3 different sites slightly increased from 73.5 ± 9.5 to 105.5 ± 16.0 ng/200 mg stool. After 24 hours on ice it was essentially unchanged at 68.1 ± 8.1 ng/200 mg stool.

This real-time PCR assay was used to quantitate *O. formigenes* levels in a total of 146 stool aliquots from 49 fecal collections from 10 subjects. The table shows the quantification of *O. formigenes* in stool samples. All stool aliquots were positive in subjects 1, 3 and 8. Only 42 of the 107 stool aliquots (38%) from the remaining 7 individuals (subjects 2, 4 to 7, 9 and 10) contained detectable levels of *O. formigenes*. Subject 5 did not have any *O. formigenes* detected. Reevaluation of the screening sample using the real-time PCR technique demonstrated that colonization was at the lower limit of detection (5×10^3 cells per gm stool).

Figure 3 shows changes in *O. formigenes* and stool oxalate during 5 days in the 4 individuals with the most consistent colonization. Subjects 1 and 3 had high colonization with levels of 3.81×10^6 to 1.04×10^9 cells per gm stool. Subject 2 had low to moderate colonization with levels of no detectable *O. formigenes* (less than 5×10^3 cells per gm) to 2×10^6 cells per gm stool. Subject 4 consistently shed low levels of *O. formigenes* in stool samples with 1 of the 15 samples containing no detectable *O. formigenes*.

The total oxalate content of stool samples was 0.1 to 1.8 mg/gm and the fecal water oxalate content was 50 to 630 μ M. On regression analysis there were no significant correlations between *O. formigenes* levels and total stool or fecal water oxalate ($p = 0.10$ and 0.83 , respectively).

DISCUSSION

The real-time PCR assay that we developed permitted determination of the levels of *O. formigenes* and its distribution within stool. This technique was used to quantify the levels of a large variety of organisms and it has advantages over other methods of detection.¹³ For example, the medium for the roll tube technique is not commercially available and colonies require 10 to 14 days to develop. The real-time PCR technique is rapid, requiring less than 1 day. In addition, it is precise based on an intravariability coefficient of variation of 1.3% for C_t and 14% for the number of bacteria enumerated, accurate based on the recovery of 96% to 110% of *O. formigenes* DNA added to an *O. formigenes* negative stool and specific based on our bacterial panel testing.

The stability of *O. formigenes* DNA was also established. Samples could be returned to the laboratory within 24 hours of passage without the loss of *O. formigenes* DNA. This stability is supported by a report of the maintenance of oxalate degradation rates in stool samples stored under similar conditions.⁹

We also observed variability in the *O. formigenes* content of stool samples among individuals and in stool samples obtained from the same individual on different days. *O. formigenes* levels varied 3 to 40,000-fold between individuals and 2 to 1,000-fold within individuals. We also detected variability of *O. formigenes* content in the same stool sample. These results are

consistent with the nonuniform distribution of organisms in fecal samples that was previously recognized in swine.^{14,15}

Lower levels of colonization were detected in this study compared to those in previous reports. The lowest levels reported by Allison⁹ and Doane⁸ et al were 4×10^6 organisms per gm, while most subjects in this study had lower levels. We recognize that this disparity could be due to differences in the populations assessed, dietary differences and the quantification techniques used.

These results also indicate that there is a significant chance of not detecting *O. formigenes* in stool specimens of colonized individuals. The organism was detected in all stool samples in only 3 subjects in this study. These results suggest that large or frequent samples of stool must be assayed to confirm the colonization status in some individuals or a pre-sample strategy should be developed to expand the stool number of *O. formigenes*. The ingestion of an oxalate rich food 1 to 2 days before testing is 1 such possible strategy. Support for this approach was provided by Doane et al, who reported that after 2-week equilibration on a spinach containing diet (1,570 mg oxalate per day) *O. formigenes* fecal levels increased 5 to 14-fold over baseline (100 mg oxalate per day).⁸

A number of factors may be responsible for the variable elimination of this bacterium in stool and the lack of correlation with stool oxalate. These factors include the soluble oxalate concentration near the mucosal wall where *O. formigenes* colonies may reside, the amount of oxalate in the diet, the bioavailability of food derived oxalate, unfavorable environmental conditions, including pH, the presence of formate or other growth inhibitory intestinal components and the inhibitory properties of other organisms. An additional factor that could contribute to the lack of a correlation of *O. formigenes* levels with stool oxalate is the presence of other fecal organisms that can degrade oxalate, including lactic acid bacteria.¹⁶

This research has potential clinical implications due to the possible effects of intestinal *O. formigenes* levels on oxalate handling in the body. Subjects with calcium oxalate stone disease colonized with *O. formigenes* were reported to have a lower urinary oxalate excretion than that of noncolonized stone formers.^{17,18} Increased urinary oxalate excretion is a risk factor for the formation of such stones. Experiments in animal models also support a role for *O. formigenes* for decreasing urinary oxalate excretion following colonization with *O. formigenes* or its daily administration.^{6,19} Colonization with *O. formigenes* may promote intestinal oxalate secretion and it is a potential therapeutic secretory route for oxalate in hyperoxalemia disorders, such as primary hyperoxaluria.^{4,5}

The exact role that an accurate enumeration of *O. formigenes* in stool samples would have in the clinical management of stones is unclear at this time. However, the enumeration of *O. formigenes* may be important for defining the relationships among intestinal levels of *O. formigenes*, dietary oxalate, fecal oxalate, other intestinal flora and intestinal oxalate absorption. Such investigations should include the study of individuals consuming diets controlled in oxalate, calcium and other nutrients to clarify these relationships. When these relationships have been defined, it will be possible to determine whether colonization with *O. formigenes* has a role in the development of stone disease.

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Abbreviations and Acronyms

C_t	threshold cycle
NTC	no template control
PCR	polymerase chain reaction

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Fig. 1. Standard curve of number of *O. formigenes* in samples vs C_t. Curve was generated by serial dilution of DNA extracted from *O. formigenes* culture, in which cell numbers were estimated by flow cytometry and DNA quantified using spectrophotometer (1 ng DNA = 4.82×10^5 cells).

Fig. 2.

Recovery of *O. formigenes* DNA added to fecal samples from noncolonized individual. Recovery rate was 96% to 110%. Dashed line represents complete recovery. Solid line represents experimental results.

Fig. 3.

O. formigenes, total oxalate and fecal water oxalate in stool samples collected from subjects 1 to 4 (A to D) during 5 days. Bars represent mean with range determined in 3 samples from different sites of same stool sample. *LOD*, limit of detection.

Table 1 Quantification of *O. formigenes* DNA by real-time PCR from stool samples of 10 individuals for 5 days

Subject No.	% Pos Fecal Aliquots*	Mean <i>O. Formigenes</i> Cells/gm Stool (range)
1	100	2.12×10^8 (4.93×10^6 – 1.04×10^9)
2	87	2.56×10^5 (less than 5×10^3 – 1.94×10^6)
3	100	7.15×10^7 (3.81×10^6 – 1.84×10^8)
4	93	1.23×10^4 (less than 5×10^3 – 4.16×10^4)
5	0	less than 5×10^3
6	27	5.80×10^3 (less than 5×10^3 – 1.7×10^4)
7	20	6.05×10^3 (less than 5×10^3 – 2.08×10^4)
8	100	5.84×10^3 (5×10^3 – 1.21×10^4)
9	7	5.02×10^3 (less than 5×10^3 – 5.3×10^3)
10	33	5.20×10^3 (less than 5×10^3 – 6.84×10^3)

Each subject provided 1 fecal sample per day and 3 aliquots from different regions of each stool were assayed.

* Greater than limit of detection of 5×10^3 cells per gm stool.